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THE ACTION OF CERTAIN STIMULATING AND INHIBITING SUBSTANCES ON THE RESPIRATION OF ACTIVE AND BLOCKED EGGS AND ISOLATED EMBRYOS[1]

(Three figures)

JOSEPH HALL BODINE AND EDGAR JOHN BOELL
Zoölogy Laboratory, State University of Iowa

IN A recent publication (Bodine and Boell, 1936*a*) attention was directed to the importance of considering the reactions of the egg as a whole, as well as those of the contained embryo, in studies dealing with the various problems in the field of developmental and cellular physiology. Such precautions seem especially applicable when definite and concrete information concerning the responses of the embryo and cells is desired.

The present study deals with results of further investigations into the responses of the entire egg, as well as those of the contained embryo, and, in addition, treats of the reactions to various stimulating and inhibiting reagents of normal, developing, and active embryos as well as those blocked or in a state of diapause. The eggs of the grasshopper, *Melanoplus differentialis*, have been used throughout; and the reader is referred to former publications for details as to procedures followed, etc., in work with such forms (Bodine and Boell, 1936*a*).

METHODS

Oxygen uptake was measured by the Warburg and microdifferential manometers at a constant temperature of 25° C. (see Bodine and Boell, 1936*a*). Embryos were dissected out in normal Belar solution under a widefield binocular microscope and thoroughly washed to free them of adhering egg materials. Oxygen determinations were carried out on groups of eggs and embryos suspended in Belar; and it was found that, after careful selection of embryos for exact morphological stages, they could conveniently be used for each determination in lots of 100. Results obtained with this number checked closely with those when embryos were used in smaller groups (10, 25, 50). Dry weights of embryos were found by drying in an electric oven at 95° C. until constant weight was attained. In all results, unless otherwise noted, values are for Q_{O_2}, or cubic millimeters of oxygen consumed by 1 mg. dry weight of embryo per hour.

Details as to methods employed in experiments with carbon monoxide, cyanide, and

[1] Aided by grant from the Rockefeller Foundation for work on cellular physiology.

methylene blue are similar to those already described (Bodine and Boell, 1934, 1936*b*), unless otherwise indicated.

METABOLIC RATES OF EMBRYOS DURING PREDIAPAUSE, DIAPAUSE AND POSTDIAPAUSE STAGES

During the course of development of the grasshopper embryo at constant temperature (25° C.), a resting, or diapause, stage occurs at about the middle of the developmental period (Slifer, 1932). This rest, or block to development, has been previously described (Bodine, 1932) and needs no further comment here except to point out that it is possible to obtain embryos in stages of development that are quite indistinguishable morphologically and at the same time are physiologically quite different. In the prediapause and postdiapause stages, cell division and growth take place; while in the diapause, cell division and growth are completely absent or blocked. In such morphologically identical embryos the rates of oxygen uptake are decidedly different, as indicated below. Differences in O_2 consumption rates have been already pointed out for eggs in these various

TABLE 1

A COMPARISON OF THE METABOLIC RATES OF GRASSHOPPER EMBRYOS DURING PREDIAPAUSE, DIAPAUSE, AND POSTDIAPAUSE STAGES WHEN THE EMBRYOS ARE MORPHOLOGICALLY INDISTINGUISHABLE

			Q_{O_2}					
Prediapause	Diapause					Postdiapause		
1.67	0.37	0.47	0.55	0.61	0.76	1.00	1.50	1.70
1.64	0.31	0.48	0.59	0.68	0.80	1.11	1.73	1.25
1.67	0.39	0.47	0.57	0.60	0.88	1.28	1.88	1.39
1.49	0.37	0.46	0.56	0.63	0.87	1.33	1.93	1.41
1.14	0.39	0.46	0.53	0.67	0.84	1.43	1.22	1.41
——	0.45	0.46	0.50	0.77	0.91	1.50	1.34	1.35
1.52 av.	0.44	0.43	0.57	0.72	0.96	1.71	1.44	1.38
	0.48	0.48	0.51	0.71	1.07	1.86	1.56	1.40
	0.41	0.45	0.69	0.72	——	1.97	1.73	1.44
	0.41	0.48	0.60	0.73	0.59 av.	1.19	1.87	——
	0.41	0.59	0.65	0.75		1.24	1.28	1.47 av.
	0.44	0.56	0.63			1.39	1.36	
			0.65			1.46	1.47	

stages (Bodine and Boell, 1936*a*), while the following observations deal almost exclusively with the naked embryos dissected out and freed from the egg contents. The marked morphological similarities of embryos at these stages is further indicated by their average dry weights: e.g., prediapause, 0.069 mg.; diapause, 0.075 mg.; postdiapause, 0.078 mg. per embryo.

Table 1 contains typical results for the Q_{O_2} of embryos in the three physiological stages. From an inspection of this table it will be noted that the average Q_{O_2} values for the prediapause and postdiapause (or the actively developing embryos) stages are 1.52 and 1.47, respectively. Variations in the Q_{O_2} values are doubtless due to inherent differences in individual lots of embryos. The average Q_{O_2} value for diapause embryos, on the other hand, is 0.59, almost three times lower than that for similar embryos when in the active developing states. The variations observed in the Q_{O_2} values for diapause embryos seem related to the potency or duration of the developmental block, since higher Q_{O_2} values are almost always associated with the onset of the breaking-up of the condition. Some ideas as to the possible working of the block mechanism have been previously expressed (Bodine, 1932).

EFFECTS OF CARBON MONOXIDE AND OXYGEN LACK ON OXYGEN CONSUMPTION RATES OF EGGS AND ISOLATED EMBRYOS

In previous reports (Bodine and Boell, 1934) it was pointed out that the physiological state of the developing egg seemed to determine the nature of its response to CO; the respiration of the actively developing egg was depressed while that for the blocked or diapause one seemed unaffected, and in certain cases even a stimulation was noted

TABLE 2

A SUMMARY OF THE EFFECTS OF CARBON MONOXIDE AND OXYGEN LACK ON DEVELOPING AND BLOCKED GRASSHOPPER EGGS

(Respiration in each case expressed in terms of percentage of normal value in air. Prediapause and postdiapause developing eggs considered in one group, since no difference in the response of the two groups to oxygen lack or carbon monoxide can be noted)

Gas Ratio	CO/O_2		N_2/O_2		O_2 Tension of Gas Mixture
	No. of Experiments	Percentage of Respiration	No. of Experiments	Percentage of Respiration	
	Developing Eggs				
50/50	.....	.....	2	116	380
70/30	6	98	.....	.....	228
75/25	6	83	.....	.....	190
80/20	69	69	157	100	152
85/15	.....	.....	6	125 (?)	114
90/10	42	49	6	72	76
95/5	10	25	8	39	38
97/3	.....	.....	2	29	23
	Diapause Eggs				
50/50	2	147	2	118	380
75/25	6	104	.....	.....	190
80/20	19	104	63	98	152
85/15	6	77	.....	.....	114
90/10	16	76	2	59	76
95/5	8	42	2	51	38

when mixtures of low CO tension and high O_2 tension were employed. These results seemed of sufficient interest to warrant their repetition, and especially with more carefully and better controlled materials and methods. Reference to Table 2 and Figure 1 shows that essentially similar results have again been found. Stimulation in the case of the diapause egg is not caused by the absorption of CO by KOH as suggested by Warburg (1928), since in each CO experiment a blank manometer was used to determine the extent of CO absorption occurring.

Comparable results for the effect of CO and O_2 lack on naked embryos are given in Table 3 and Figure 1. As in the case of the whole egg, a marked difference in the re-

sponse to CO is obtained for developing and diapause embryos. The diapause embryo is invariably much less sensitive to CO than the developing one. For O_2 lack, no significant differences seem indicated.

In general the response of the naked embryo to O_2 lack and CO is less marked than that for the intact egg. Further investigations are being carried out with the hope of explaining the curious stimulating effects of CO on the O_2 uptake of both blocked embryo and egg.

EFFECT OF POTASSIUM CYANIDE ON RESPIRATION OF EGGS AND NAKED EMBRYOS

Previous experiments have indicated marked differences in susceptibility to KCN for developing and blocked eggs (Bodine, 1934); Bodine and Boell, 1934. In the present work a repetition of these experiments has been made with a more careful check upon the experimental conditions involved. The KCN was neutralized to pH 7.0 with HCl and diluted immediately before use. The CO_2 absorbing mixture was made up of one part of 1.82 M KCN plus one part 0.182 M KOH. This KCN-KOH mixture has the same HCN tension as the solution surrounding the eggs, and hence no absorption of HCN gas by the KOH should occur. In Table 4 are given data typical of the results found for eggs. For the concentration of cyanide (1×10^{-3} M) most extensively used, the average residual respiration for the developing egg is 26 per cent, while that for the diapause one is 81 per cent. This marked difference in cyanide sensitivity for the two physiological states of the eggs is similar to that previously indicated (Bodine, 1934; Bodine and Boell, 1934). By using slightly lower concentrations of the reagent, it is possible to obtain marked depression of the developing egg and scarcely any effect whatever on the diapause or blocked one.

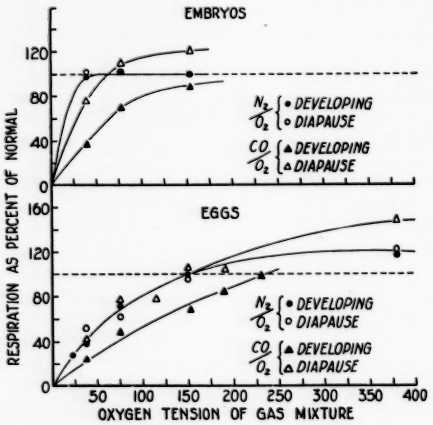

FIG. 1.—Showing effect of oxygen lack and carbon monoxide on the oxygen uptake of developing and blocked eggs and isolated embryos. Experimental conditions similar in all determinations. Each point is an average of several experiments. For further description see text.

The action of cyanide on the naked embryo, however, is quite in contrast with that found for the egg. Using the same techniques in which HCN absorption was prevented, a true residual respiration for the embryo has been obtained, as shown in Table 5, where results, typical for the effects of different cyanide concentrations, are listed. Marked differences in sensitivity to the cyanide by the active and blocked embryo are not as evident as for the egg. In many cases, however, it can be shown that the blocked embryo tends to be the more resistant. Considerable variation exists for the blocked embryos or different lots, and in certain cases no differences between the resistance of the blocked and active embryos to the reagent are noted.

A possible explanation for these differences in response of the egg and embryo is that the yolk cells, heretofore considered more or less inert, contribute much to the general

TABLE 3

SUMMARY OF THE EFFECTS OF OXYGEN LACK AND CARBON MONOXIDE ON DEVELOPING AND BLOCKED GRASSHOPPER EMBRYOS

(Respiration in each case expressed as percentage of the normal value in air)

Gas Ratio	CO/O_2		N_2/O_2	
	No. of Experiments	Percentage of Respiration	No. of Experiments	Percentage of Respiration
	Developing Embryos			
50/50	.....	.....	1	128
80/20	12	90	47	100
90/10	13	72	5	105
95/5	9	37	5	95
97/3	.....	.....	2	82
	Diapause Embryos			
80/20	8	122	33	100
90/10	9	109	4	103
95/5	8	77	4	101

TABLE 4

SUMMARY OF THE EFFECT OF 1×10^{-3} M POTASSIUM CYANIDE ON THE RESPIRATION OF DEVELOPING AND BLOCKED EGGS

(Oxygen uptakes expressed as cubic millimeters of oxygen consumed per hour by 100 eggs. Age of developing eggs refers to days of postdiapause development. Age of diapause eggs denotes the number of days from the time of laying.)

Developing Eggs				Diapause Eggs			
Age	O_2 Uptake		Percentage of Control	Age	O_2 Uptake		Percentage of Control
	Control	In 1×10^{-3} M KCN			Control	In 1×10^{-3} M KCN	
1	48.3	13.3	27	37	9.0	5.3	62
1	49.4	13.2	27	37	8.0	5.9	69
2	38.8	11.5	30	38	8.9	9.8	105
5	75.3	19.2	26	38	9.7	6.1	66
5	74.0	21.6	29	53	8.6	7.6	94
7	86.0	17.3	20	53	7.6	7.3	90
7	83.0	16.6	20				
8	85.0	25.0	29	Av.	.....	.....	81
8	89.0	20.0	23				
Av.	.....	.....	26				

physiological response of the intact egg (Bodine and Boell, 1936*a*). Variations in the quantitative responses to cyanide are also doubtless due to inherent physiological conditions in different lots of eggs and embryos, as well as to possible physiological states at the time of exposure to the reagent. A more extensive and detailed investigation of these points is being carried out.

TABLE 5

SUMMARY OF THE EFFECT OF CYANIDE ON THE RESPIRATION OF DEVELOPING AND BLOCKED EMBRYOS

Age of Embryos	Molar Concentration of KCN	Q_{O_2}		Q_{O_2} in KCN as per Cent
		Control	In KCN	
	Developing Embryos			
2	1×10^{-4}	1.22	0.59	45
2	1×10^{-4}	1.39	.....	.....
1	1×10^{-3}	2.21	0.24	11
2	1×10^{-3}	1.36	0.06	4
5	1×10^{-3}	0.59*	0.02*	4
7	1×10^{-3}	0.95*	0.07*	7
Average residual	.....	.....	.....	9
	Diapause Embryos			
65	5×10^{-4}	0.65	0.27	41
38	1×10^{-3}	0.67	0.05	7
53	1×10^{-3}	0.45	0.11	25
98	1×10^{-3}	0.48	0.07	14
98	1×10^{-3}	0.50	0.06	12
98	1×10^{-3}	.....	0.05	10
Average residual	.....	.....	.....	14

* These values represent respiration as cubic millimeters of O_2 per embryo per hour, not Q_{O_2}. Adequate weight data for older postdiapause embryos has not yet been obtained and Q_{O_2} can, therefore, not be accurately expressed.

ACTION OF METHYLENE BLUE, METHYLENE BLUE PLUS CYANIDE, AND METHYLENE BLUE PLUS CARBON MONOXIDE ON THE RESPIRATION OF ISOLATED EMBRYOS

Some indications of the action of methylene blue on the O_2 uptake of active and blocked embryos have been presented in a preliminary report (Bodine and Boell, 1936*b*). More detailed and extensive series of experiments have been carried out, the results of which are given below. In Table 6 and Figure 2 are shown data which indicate that for developing or active embryos the maximum stimulation due to the addition of methylene blue is relatively small. With different concentrations of the reagent the amount

TABLE 6

CATALYTIC ACTION OF METHYLENE BLUE ON THE RESPIRATION OF DEVELOPING EMBRYOS

Age	Molar Concentration of Methylene Blue $\times 10^{-5}$	Q_{O_2}		Percentage of Increase
		Control	Methylene Blue	
6*	3.1	0.61	0.56	− 8
21†	3.1	1.14	1.16	2
21†	3.1	.....	1.11	− 2
2	3.1	1.36	1.68	23 Av. 4
1	6.3	1.28	1.65	31
1	6.3	1.24	1.64	30
1	6.3	.....	1.60	27
2	6.3	1.22	1.69	30
2	6.3	1.39	1.68	29 Av. 29
1	9.4	1.34	1.61	16
1	9.4	1.41	1.85	34
1	9.4	.....	1.85	34
1	9.4	1.50	1.71	20
1	9.4	1.33	1.66	17
1	9.4	1.41	1.54	8
1	9.4	1.43	1.68	18
1	9.4	.....	1.47	3 Av. 19
1	12.6	1.39	1.82	23
1	12.6	1.56	2.16	46
			1.76	19 Av. 29
Av.	.....	1.36	1.65	19

* Postdiapause embryo for which respiration is expressed as cubic millimeters of O_2 per embryo per hour. All other values of respiration in table are Q_{O_2} values.

† Prediapause embryos.

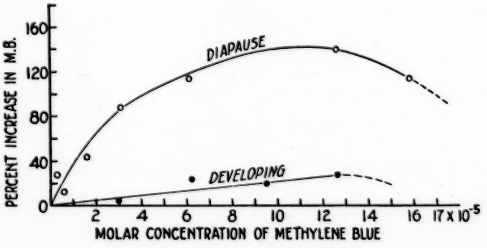

FIG. 2.—Showing effect of methylene blue on the oxygen uptake of active and blocked isolated embryos. Each point is an average of several experiments. For further description see text.

of stimulation increases, gradually reaching a maximum at approximately 12.6×10^{-5} M. For the diapause or blocked embryo (Table 7, Fig. 2), on the other hand, the

TABLE 7

CATALYTIC ACTION OF METHYLENE BLUE ON THE RESPIRATION OF BLOCKED EMBRYOS

Age	Molar Concentration of Methylene Blue $\times 10^{-5}$	Q_{O_2}		Percentage of Increase	
		Control	In Methylene Blue		
109	0.31	0.68	0.86	27	
42	0.63	0.76	0.85	12	
109	1.57	0.68	0.97	43	
109	3.1	0.68	1.02	50	
42	3.1	0.76	1.39	83	
34	3.1	0.63	1.26	121	
34	3.1	0.51	1.20	110	
38	3.1	0.67	1.29	94	
82	3.1	0.61	.....	.....	
82	3.1	0.51	0.93	83	
82	3.1	0.47	0.77	65	Av. 87
109	6.3	0.68	1.33	96	
84	6.3	0.45	1.10	125	
84	6.3	0.53	0.98	100	
84	6.3	.....	1.07	119	
57	6.3	0.57	1.39	91	
57	6.3	0.65	1.59	118	
57	6.3	0.84	1.45	99	
57	6.3	0.85	1.59	118	
68	6.3	0.37	0.77	83	
68	6.3	0.48	1.20	186	
68	6.3	.....	1.19	183	
65	6.3	0.65	0.97	49	Av. 114
100	12.6	0.57	1.35	132	
100	12.6	0.60	1.43	146	Av. 139
42	15.7	0.76	1.56	106	
98	15.7	0.48	1.19	143	
98	15.7	0.50	1.18	141	
108	15.7	0.73	1.41	90	
108	15.7	0.75	1.66	124	
108	15.7	.....	1.47	98	Av. 117
Av.	.....	0.62	1.23	109	

degree of stimulation is greatly in excess of that for the active one. Here, too, the effects of different concentrations of the reagent are clearly indicated, reaching a maximum at approximately 12.6×10^{-5} M. It is of some importance to emphasize again that all embryos used were morphologically similar, the marked differences being that the

physiological states of the cells composing them are at markedly different levels. Methylene blue in all cases produces a greater stimulation in the rates of O_2 uptake in the cell while in the blocked condition, thus suggesting that perhaps the physiological state of the cell is of much importance in determining its response to such stimulating compounds (see Bodine and Boell, 1934, 1936*b*).

Inasmuch as marked antagonism of cyanide by methylene blue has been pointed out by various workers (Barron, 1929; Barron and Hamburger, 1932; Brooks, 1932*a*, 1932*b*,

TABLE 8

ACTION OF CYANIDE ON RESPIRATION OF EMBRYOS IN PRESENCE OF METHYLENE BLUE
(Concentrations of methylene blue and cyanide represent final concentrations in the manometer vessel during the experiment)

Age	Molar Concentration of KCN $\times 10^{-3}$	Molar Concentration of Methylene Blue $\times 10^{-5}$	Q_{O_2}						
			Control	KCN	Per Cent	KCN + Methylene Blue	Per Cent	In Methylene Blue	Per Cent
	Developing Embryos								
2	0.1	6.3	1.22	0.59	45	1.92	147	1.69	130
2	0.1	6.3	1.39	.....	.....	1.45	111	1.68	129
2	1	3.1	1.36	0.06	4	0.26	19	1.68	123
	Diapause Embryos								
65	0.50	6.3	0.65	0.27	41	1.01	155	0.97	149
38	1	3.1	0.67	0.05	7	0.25	38	1.29	194
98	1	15.7	0.48	0.07	14	0.35	72	1.19	243
98	1	15.7	0.50	0.06	12	0.33	68	1.18	241
98	1	15.7	.....	0.05	10	0.26	53	.....	.....

1932*c*, 1933), it was thought desirable to investigate these phenomena with embryos in different physiological states. In Table 8 are shown data thus obtained. With the use of appropriately low concentrations of cyanide, e.g., 1×10^{-4} M, practically complete reversal of the cyanide inhibition can be accomplished by the addition of methylene blue. This result is similar to that reported earlier by Bodine and Boell (1936*b*). However, when the respiration of embryos has been depressed by concentrations of cyanide of the order of 1×10^{-3} M, or stronger (dosages which still permit of a readily reversible effect), the addition of methylene blue causes only a slight, and perhaps insignificant, increase in respiration. A comparison of columns 8 and 10 of Table 8 indicates that the respiration of both blocked and developing embryos is much lower in the presence of

cyanide plus methylene blue than it is in the presence of methylene blue alone. Thus, not only the normal respiration of the embryos, but also that which is stimulated by methylene blue, is cyanide sensitive.

The results obtained with the grasshopper embryo, therefore, stand in striking contrast to those reported by Barron (1929) for the sea-urchin egg. With this material, respiration which had been totally inhibited by 0.01 M cyanide was restored completely to the normal level through the addition of approximately 15×10^{-5} M methylene blue. Barron and Hamburger (1932) likewise reported marked antagonism by toluylene blue of the depressing effect of cyanide on the respiration of echinoderm and annelid eggs. In their experiments the respiration in the presence of dye plus cyanide was, in many cases, practically equal to the respiration in the presence of the dye alone.

The action of methylene blue plus CO on the developing and blocked embryo has also been studied. It will be recalled that CO has little effect on the blocked embryo when O_2

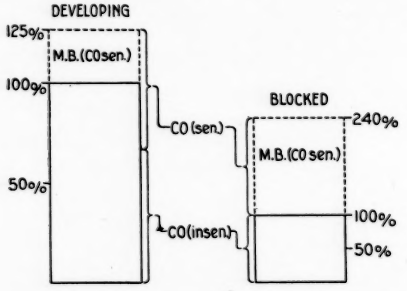

FIG. 3.—A schematic representation of the action of methylene blue and carbon monoxide on the respiratory mechanisms of the developing and blocked embryo. Relative values of oxygen uptake indicated by height of columns. Percentages shown are taken from data of experiments. Symbols: *CO* refers to carbon monoxide sensitive (sen.) or insensitive (insen.) fractions of respiratory mechanism: *M.B.*, that fraction resulting from stimulating effect of methylene blue. For further description see text.

deficiency is not a factor, while the active embryo under the same conditions is markedly affected, as evidenced by a decided drop in its rate of O_2 uptake (Fig. 1). Methylene blue, added to the CO-depressed active embryo, has little, if any, effect in actively antagonizing the depressing action of the CO, as indicated in results of experiments in Table 9 (see Fig. 3).

If to the blocked embryo, however, methylene blue is added, its rate of O_2 uptake, as indicated above, is markedly increased (Fig. 2). Carbon monoxide added to the methylene-blue-stimulated blocked embryo invariably produces a marked reduction in the O_2 uptake of the methylene-blue-stimulated fraction, as indicated in Table 10 and Figure 3. It is of interest to note that a blocked embryo, even though not normally depressed by CO, can become so if the O_2 uptake is increased by the addition of methylene blue. At the same time, attention must be focused upon the fact that subsequent additions of CO to the methylene-blue-stimulated embryo result only in the depression of the methylene-blue-stimulated fraction of the O_2 uptake. The similarity of the action of CO on the methylene-blue-stimulated fraction of the blocked embryo and that of the normal active embryo is of much interest and is perhaps indicative of a likeness in the

TABLE 9

SHOWING SENSITIVITY OF RESPIRATION OF DEVELOPING EMBRYOS TO CARBON MONOXIDE IN PRESENCE OF METHYLENE BLUE

(Experiments 1 and 2: respiration expressed as cubic millimeters of oxygen consumed per hour per embryo. Concentration of methylene blue used in these experiments, 3.1×10^{-5} M. Experiments 3 and 4: concentration of methylene blue, 9.4×10^{-5} M.)

Expt.	Age	CO/O_2	Q_{O_2}					Increase Due to Methylene Blue in Presence of CO (Per Cent)
			Control	CO/O_2	Per Cent	Methylene Blue Plus CO	Per Cent	
1	6	90/10	0.61*	0.38*	63	0.39*	64	1
2	6	90/10	.....	.....	.....	0.36*	59	−4
3	1	95/5	1.41	0.39	27	0.43	30	3
4	1	95/5	1.43	0.37	26	0.43	30	4

* Respiration as cubic millimeters of O_2 per embryo per hour.

TABLE 10

ACTION OF CARBON MONOXIDE ON RESPIRATION OF BLOCKED EMBRYOS IN PRESENCE OF METHYLENE BLUE

(Figures in parentheses indicate percentages in terms of average control as 100 per cent)

CO/O_2, N_2/O_2 } = 95/5

Methylene blue = 12.6×10^{-5} M

Q_{O_2}					
Control	Methylene Blue	CO/O_2 95/5	N_2/O_2 95/5	CO Plus Methylene Blue	N_2/O_2 Plus Methylene Blue
0.52(102)	0.91	.....	.....	.....	.....
0.53(104)	.....	0.29(57)	.....	0.60(118)	.....
0.46(90)	.....	0.35(69)	.....	0.57(112)	.....
0.53(104)	.....	.....	0.51(100)	.....	0.93(185)
0.53(104)	.....	.....	0.53(104)	.....	0.86(169)
Av. 0.51 (100)	0.91 (179)	0.32 (63)	0.52 (102)	0.58 (114)	0.90 (177)

respiratory mechanism active in the two cases. In a subsequent report, additional data as to the possible mechanisms involved will be presented.

DISCUSSION

It is of some interest to note again that observations carried out on the intact egg are not of themselves sufficient evidence to warrant their being carried over directly to the contained embryo. The developing egg is a system, one member of which, in the case of the grasshopper egg, is the embryo. The relative physiological importance of the members of such systems, as yolk cells, membranes, etc., is difficult to evaluate; but it seems reasonable to suppose that, for a period at least, the embryo proper plays but a minor role in the general metabolic or chemical activity of the egg.

The relatively simple arrangement of the embryo proper in relation to the extra-embryonic parts of the grasshopper egg makes such material extremely favorable for the separation and study of the component parts. The inherent diapause or block enables embryos of the same morphological stages, but with different physiological properties, to be investigated without such disturbing factors as differences due to unequal size, etc. Data for the O_2 consumption rates of the embryo can thus be qualitatively and quantitatively compared under many different sets of experimental conditions. Quantitative differences between the reactions of the egg and contained embryo are of peculiar interest because they make possible an analysis on a definitely cellular basis rather than on one based solely upon the egg.

The differences in the response to methylene blue exhibited by blocked and developing embryos seem to indicate that methylene blue apparently can produce an appreciable stimulation of O_2 uptake only when, as in the case of embryos during diapause, part of the normal respiratory activity is blocked. Methylene blue may thus be considered as substituting, to some extent at least, for that part of the respiratory mechanism which is blocked during diapause. With the gradual weakening of the block or a loss in its potency, a corresponding decrease in the methylene blue sensitivity invariably follows. It seems reasonable to expect that further studies along these lines may possibly give clues as to the probable workings of the so-called "block" mechanism.

SUMMARY AND CONCLUSION

1. Oxygen consumption rates have been determined for morphologically identical, but physiologically different, stages of the embryo of the grasshopper, *M. differentialis*. Rates have been expressed in terms of O_2 uptake per milligram dry weight per hour. Embryos in the active developmental stages have O_2 rates approximately three times greater than similar embryos when in the blocked or diapause stage.

2. Oxygen lack and CO affect the O_2 uptake of active and blocked isolated embryos in a way essentially similar to that for the intact eggs. The blocked or diapause embryo is quite resistant to the action of CO; the active embryo, on the other hand, is quite susceptible.

3. The action of KCN on the O_2 uptake of active and blocked eggs has been investigated. The blocked or diapause egg is found to be markedly cyanide resistant, thus confirming previous results. The isolated embryos from both active and blocked eggs show considerable variation in their resistance to cyanide; they are both quite cyanide sensitive, although in certain cases the blocked or diapause embryo is the more resistant.

4. Methylene blue stimulates the O_2 uptake of the blocked or diapause embryo to a marked degree. The active or developing embryo is only slightly sensitive to methylene blue; differences in the action of methylene blue on the embryos seem to be related to their physiological states.

Antagonism by methylene blue of the depressing effect of cyanide on respiration is apparent only when relatively low concentrations of cyanide are used. The presence of methylene blue does not prevent the depression of respiration by CO in developing embryos. In blocked embryos the increased O_2 consumption induced by the dye can be completely restricted by CO.

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EFFECTS OF ROENTGEN RADIATION ON EMBRYONIC ORGANIZATION AND REGULATION IN MELANOPLUS DIFFERENTIALIS (ORTHOPTERA)[1]

(Seven figures)

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THE writer has previously reported (1936) the finding of several abnormal embryos in irradiated eggs of the grasshopper *Melanoplus differentialis*. These anomalies were not merely injuries or defects but were definite alterations of the normal organization. It is the purpose of this paper to report the results of an investigation to determine whether the abnormalities could be produced in large enough numbers and in definiteness of character sufficient to permit a study of their origin and significance.

MATERIALS AND METHODS

Eggs of *M. differentialis* were collected on the day laid and were incubated at 25° C. The eggs were irradiated at room temperature and then immediately placed back in the incubator. The Roentgen-ray machine is a Coolidge, air-cooled, tungsten-target, broad-focus tube with mechanical rectification. The tube was activated at 130 kv. and 5 ma. The organisms were suspended on a cloth 30 cm. from the target of the tube. The intensity was measured by means of a commercial dosimeter, and was usually 200 roentgens per minute. The reader is referred to the papers of Bodine (1932), Slifer (1932), and Evans (1934, 1935, 1936) for further details covering the grasshopper's development.

EXPERIMENTAL

On frequency of occurrence of abnormal development.—The first experiments were attempts to correlate the frequency of abnormal development with a given morphological stage and amount of radiation. Over 14,860 eggs have been irradiated at varying stages and examined individually at the time of diapause (21 days at 25° C.). Approximately 3,000 controls have also been examined and have been found to be very regular in morphological appearance. The writer has found only 1 morphologically abnormal embryo in all the thousands he has examined (see No. 5 of Fig. 1). Other investigators in the Zoölogy Department at the State University of Iowa have occasionally discovered such embryos in their control lots. The writer is indebted to Mr. Vincent Thompson for No. 4 of Figure 1, to Mr. Arthur Schipper for No. 2 of Figure 1, and to Mrs. Evelyn Cook for No. 3 of Figure 1.

The age of the eggs when irradiated varied from 1 to 14 days. The dosage ranged from 50 to as high as 6,600 roentgens (see Table 1 for details). Figure 1 shows that the

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frequency varies with the morphological stage (age) and with the dosage. The optimum dosage varies with the developmental stage according to the general radiosensitivity of the eggs at the different ages (see Evans, 1936, for details). Figure 2 is a graphic presentation of the dosage which results in the greatest number of aberrations at the different ages. Thus, it is shown that, for all experiments at a given dosage, the most favorable were on the seventh day. The highest percentage in a single experiment (not shown in Table 1 or in Fig. 2) was 50 following a dosage of 250 roentgens on the sixth day. It is evident that the period of development between the sixth and the seventh days is most favorable for the production of abnormal development. During this time the extensive cellular differentiation comparable to gastrulation in vertebrates is taking place. The gastrulation stage has been found to be most susceptible to irradiation in other insects (Henshaw, 1933; Scott, 1934; and Packard, 1935). Gastrulation has been found by many workers to be a period during which radiation affects differentiation in other organisms (Dugger, 1936, pp. 396–97).

FIG. 1.—Drawings of embryos at the time of diapause showing some of the abnormalities which occurred in control lots. Number 1 is normal control. Number 2 has two extra heads and mouth parts. Number 3 is a lateral twin joined at the abdomen. Number 4 is an almost complete twin back to back. Number 5 is an embryo with a double abdomen.

Another finding relates to the character of the abnormality. The anomalies found in eggs irradiated before the sixth day were local aberrations such as duplications of recognizable structures. Abnormalities of the thorax were (*a*) additional segments and appendages longitudinally, (*b*) additional appendages laterally, and (*c*) appendages split at a basal joint. Occasionally an embryo would be found with an extra abdomen posteriorly or laterally. Some abnormalities of this general type were found in eggs irradiated during the fifth to the seventh days, but these anomalies were usually limited to appendages. The abnormalities of the character indicated above have been classed as Type 1, and typical examples are shown in Figure 3.

Most of the abnormalities found in eggs irradiated on the fifth to the seventh days were outgrowths from the head and have been classified as Type 2. Figure 4 is a series of drawings of typical Type 2 abnormalities. The outgrowths distinctly arise from the frontal regions of the head and branch in many directions. The recognizable structures are apparently thoracic appendages and mouth parts. The smaller outgrowths are characteristic of effects of irradiating eggs 300–400 roentgens on the seventh and eighth days and of lighter dosages on the fifth and sixth days. At all ages suboptimal dosages result in no morphological injury, and irradiations that are too heavy result in such extensive injury as to obscure any attempts at abnormal differentiation. Figure 5 shows some embryos found in eggs rayed with heavier dosages (250–350 roentgens) on

TABLE 1

Occurrence of abnormalities at different ages (25° C.)

Dosage (Roentgens)	No. of Eggs	No. of Abnorms.	Percentage of Abnorms.
1-Day Eggs			
100	997	0	0
200	1,926	3	0.16
300	534	0	0
400	284	0	0
500	68	0	0
1,000	93	0	0
6,600	50	0	0
2-Day Eggs			
100	520	0	0
200	920	0	0
300	120	0	0
400	36	0	0
500	50	0	0
1,000	85	0	0
2,000	50	0	0
3,000	50	0	0
3-Day Eggs			
100	94	0	0
200	111	0	0
300	108	2	1.8
400	125	2	1.6
1,000	35	0	0
4-Day Eggs			
100	46	0	0
200	46	0	0
300	59	0	0
400	114	4	3.5
500	138	1	0.72
600	25	0	0
800	25	0	0
1,000	58	0	0
5-Day Eggs			
100	290	5	1.73
200	867	75	8.7
300	164	9	5.5
400	130	12	9.5
500	62	0	0

Dosage (Roentgens)	No. of Eggs	No. of Abnorms.	Percentage of Abnorms.
6-Day Eggs			
100	621	7	1.12
200	1,282	157	12.3
300	581	67	11.5
400	326	24	7.4
500	58	0	0
1,000	30	0	0
7-Day Eggs			
100	61	9	14
200	155	27	17.5
300	255	35	13.7
400	167	45	27
500	108	0	0
8-Day Eggs			
100	73	0	0
200	119	2	1.68
300	93	4	4.6
400	107	6	5.6
500	62	0	0
1,000	35	0	0
9-Day Eggs			
100	197	0	0
200	115	0	0
300	117	1	0.85
400	77	3	3.9
500	35	0	0
1,000	72	0	0
10-Day Eggs			
100	61	0	0
200	51	0	0
300	547	0	0
400	105	3	2.85
500	49	0	0
1,000	32	0	0

TABLE 1—*Continued*

Dosage (Roentgens)	No. of Eggs	No. of Abnorms.	Percentage of Abnorms.
11-Day Eggs			
100	104	0	0
200	74	0	0
300	100	0	0
400	112	0	0
500	78	0	0
1,000	94	0	0
12-Day Eggs			
100	33	0	0
200	41	0	0
300	27	0	0
400	39	0	0
500	86	0	0
1,000	57	0	0
13-Day Eggs			
100	45	0	0
200	64	0	0
300	49	0	0
400	79	0	0
500	48	0	0
1,000	59	0	0

the fifth and sixth days, and are apparently advanced stages of aberrations shown in Figure 4. These embryos shown in Figure 5 are apparently more or less successful attempts to produce second embryos. It will be noted that they are twins in the longitudinal axis and not laterally as has occurred in controls (Fig. 1).

All the embryos in the foregoing experiments were examined at the time of diapause, when the organisms were normally inactive. Diapause occurs in nearly all the eggs of this species although its duration varies greatly. Samples were taken from irradiated lots from time to time, and the appearance and growth of the aberrations were observed under the dissecting microscope. It is interesting to note that, although irradiated embryos sometimes developed for a few days after the controls were in diapause, they too ceased to grow when the morphological stage characteristic of diapause was reached. The aberrant outgrowths, also, were inactive during diapause. It thus appears from this indirect evidence that, although the differentiation of a large number of cells is altered, their ultimate development is still under some sort of general control which regulates growth.

Effects of the aberrant growths on later development.—Postdiapause development was studied by placing the irradiated prediapause and diapause eggs at a temperature of 5° C. for over 1 month and then returning them to the incubation temperature (25° C.) where they developed continuously until the time of hatching. It was found that if the aberrant growths were extensive, the embryo was unable to hatch. In fact, if the outgrowth was very large, the embryo was unable to complete yolk engulfment. When the aberrant growth was small, it was usually resorbed, and several embryos hatched with only scars marking the site of the aberration. Some typical embryos examined in postdiapause stages are shown in Figure 6.

Several lots of eggs of a closely related species, *M. bivittatus*, were irradiated on the fifth and sixth days. Most of these embryos went through blastokinesis and yolk engulfment before going into diapause, and were thus comparable in many ways to the cold-

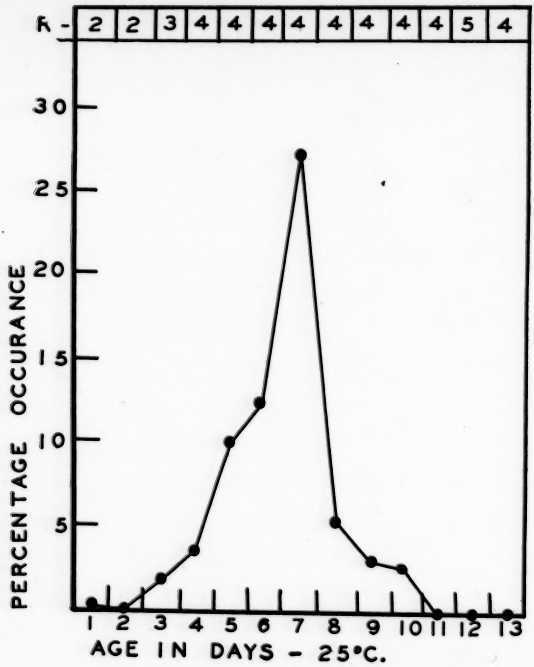

FIG. 2.—Graph showing percentage occurrence of abnormalities at different ages. Ordinates indicate the maximum occurrence in percentage of total number of eggs at a particular dosage. The dosage which gave this maximum effect is represented in *R* (×100 roentgens). The abscissas show the age of the eggs in days (25° C.).

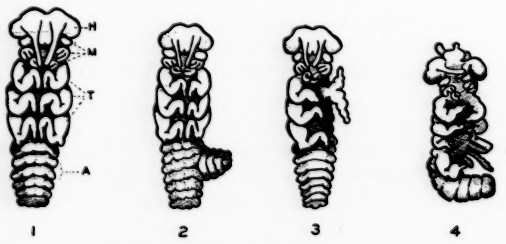

FIG. 3.—Drawings of type 1, abnormalities found in irradiated eggs. No. 1, is of a control at diapause: *H*, head, *M*, mouth parts, *T*, thorax, and *A*, abdomen. Number 2 is of an embryo from an egg rayed with 300 roentgens on the first day and shows a second abdomen formed laterally. Number 3 was irradiated with 200 roentgens on the fifth day. It will be noted that mesothoracic and metathoracic legs are missing and that the left prothoracic appendage is bifurcated. Number 4 was irradiated on the sixth day with 200 roentgens and possesses supernumerary appendages.

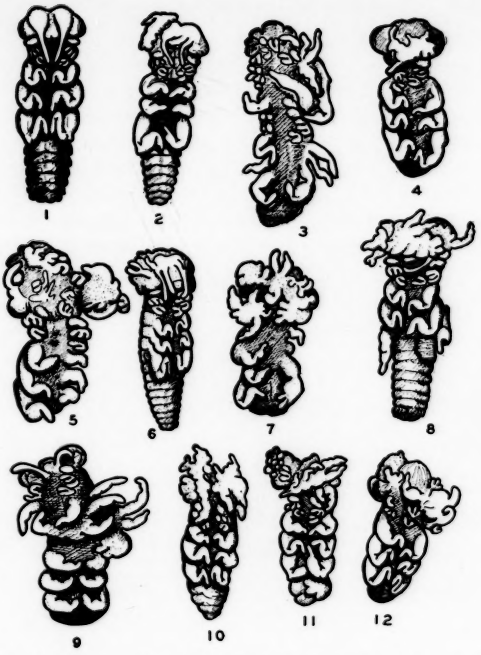

FIG. 4.—Embryos showing abnormalities of Type 2. Number 1 is of a control at the time of diapause. All eggs rayed on fifth and sixth days with from 200 to 300 roentgens.

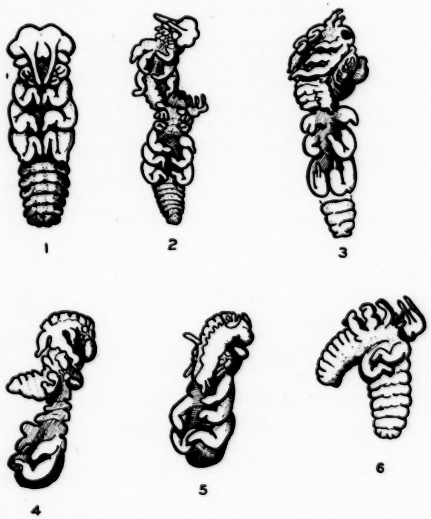

FIG. 5.—Embryos irradiated on fifth and sixth days with 240–350 roentgens. Number 1, control at time of diapause. Numbers 2, 3, 4, 5, and 6 are embryos from irradiated eggs and show longitudinal twinning.

treated eggs of *M. differentialis*. These eggs likewise gave evidence of some competition between the primary embryo and the secondary growths. Many embryos had large aberrations, and a few had developed complete secondary embryos of the stage at which *M. differentialis* goes into diapause. In all these cases the primary embryo remained small and did not complete yolk engulfment. Localized abnormalities, on the other hand, did not interfere with the development previous to hatching. Many of the smaller outgrowths were shriveled and were apparently disintegrating. It is concluded that the secondary growth may be induced even while the primary one is still growing, and during the later development the growth of small aberrations is restricted in favor of the normal structures.

FIG. 6.—Number 1, control embryo after yolk engulfment. Number 2, embryo from egg irradiated with 200 roentgens on sixth day and then exposed to cold and returned to 25° C. Aberrant growth reduced to small area on right side of head. Number 3 is of an irradiated embryo of same age. Note outgrowths on left of head and the separation of the lower ocellus into two parts. Number 4, embryo of same age as other irradiated eggs, but showing extensive outgrowths on head which prevent yolk engulfment, etc. Number 5 is an embryo so injured by radiation as not to be able to complete blastokinesis. The outgrowth on the left side has split the eye into two. Number 6 is of an embryo of *M. bivittatus* after yolk engulfment which was irradiated with 200 roentgens on sixth day and shows presence of an extra thoracic segment and appendages.

Loss of regulation during development.—It was thought that some light would be shed on the understanding of the mechanisms involved in the production of these abnormal growths if more were known concerning the differentiation and loss of regulatory powers during the early development of *M. differentialis*. Eggs of ages from 1 to 9 days were irradiated with 200 roentgens and examined at the time of diapause. There were 50 eggs to each lot irradiated and a similar number of controls. The results of these experiments are shown in Graph A of Figure 7. The eggs rayed on the first 2 days contained either complete embryos (when examined at the time of diapause) or none at all. Those rayed on the third and fourth days contained many embryos with the whole head, thorax, or abdomen missing. The ones treated on the fifth to the eighth days contained many with local defects or aberrations. It thus appears that, as eggs develop, the injury due to a certain dosage becomes more restricted, and the ability to replace injured cells likewise decreases. A group of eggs similar to the one in the above-mentioned experi-

ments was irradiated with 2,000 roentgens through a grid composed of strips of lead foil alternating with strips of photographic film. This treatment increased the dosage about ten times but allowed alternating strips of the egg to be almost unaffected. The results of this experiment are shown in Graph B of Figure 7. The examination of the eggs irradiated under these conditions made the loss of regulative powers during development even more evident. It is concluded that uninjured cells of the 1- to 3-day eggs have the power of replacing those destroyed; that during the fourth to the eighth days this power becomes more and more restricted. The change from the regulative phase

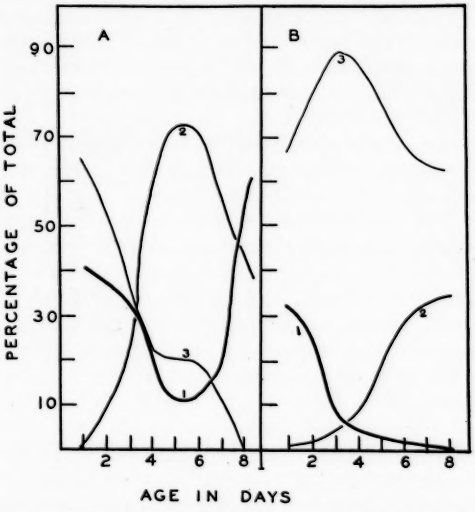

FIG. 7.—Two graphs showing loss of regulation during development. The abscissas indicate age of eggs in days (25° C.). The ordinates give the number affected in percentage of the total. In either graph curve 1 shows the percentage of eggs which contained normal unaffected embryos when examined at the time of diapause. Curve 2 represents those eggs examined at diapause which contained only partial embryos. Curve 3 shows the number of eggs in which the injury was extensive and complete. Graph A is of an experiment in which the eggs were irradiated with 200 roentgens. Graph B is of an experiment in which the eggs were irradiated with 2,000 roentgens through a grid which allowed alternating strips of the egg to be protected from the radiation.

to the nonregulative condition occurs from the fourth to the seventh days. It is significant that this is the period during which the aberrant growths appear most frequently. It seems logical to infer that these secondary outgrowths may be abortive attempts at the regeneration of a new embryo. The fact that the outgrowths originate at definite regions of the head suggests that these cells are the only ones in the embryo at this time which have retained this potentiality.

DISCUSSION

The possibility that Roentgen radiation has a direct effect on cellular differentiation must be recognized because of its ability to cause basic changes in matter and especially

in chromatin (Dugger, 1936, pp. 1167–1262). It is difficult to recognize such direct changes brought about by radiation in the embryos because of their microscopic size and because changes of the same nature are not frequent. Effects of a grosser nature such as to result in abnormal or abortive cleavages, are more easily and frequently recognized. The fact that destruction of cells has different effects on embryonic development causes one to consider the possibility that the radiation acted only indirectly on differentiation. The indirect action of the radiation probably is the selective destruction of certain cells, thus acting as an experimental knife.

In order to understand the probable mechanism of the effect of the radiation, it is pertinent to consider briefly the understanding of processes of insect development. The reader is referred to the recent paper of Howland and Sonnenblick (1936) and Brauer and Taylor (1936) for recent reviews of the literature. It may be said in general that eggs of different orders of insects vary in the degree of their regulatory powers. The Odonata are most regulative, and then follow the Orthoptera, Coleoptera, Hymenoptera, and Lepidoptera in the order named. Seidel (1926–34) has postulated a *Bildungszentrum* which is necessary for development, and which in turn produces the *Differenzierungszentrum* located in the region of the presumptive thorax which organizes the ventral anlage of the insect. The methods used to demonstrate the foregoing conclusions included cautery, puncture, ultra-violet raying, and constrictions.

Kraus (1934) has shown that the egg of the primitive orthopteran *Tachycines asynamorus* is highly regulative and loses this power only after the formation of the heart-shaped embryo. Splitting of the blastoderm while the heart-shaped embryo was being formed resulted in the production of twins and duplication of parts. About 6 hours after the formation of the heart-shaped embryo the regulation was somewhat restricted, and 18 hours after, the removal of parts always resulted in defects. The asymmetrical duplications indicated differentiation material to be centered in the first thoracic segment and to proceed thereafter to the anterior and posterior regions. The longitudinal axis is laid down first, and then the right and left sides are established.

The abnormal embryos of Type 1 of the present paper may possibly be comparable to those of Kraus in that they are not laterally symmetrical and are usually duplications of localized regions. The anomalies of Type 2, however, are apparently not the result of splitting of embryonic anlagen. In the first place, they are sometimes in the longitudinal planes, and Kraus found only lateral twinning. The two twins found in the controls of *M. differentialis* were lateral ones, and the co-twins were of the same size. This matter of size also indicates that the abnormalities of Type 2 represent a delayed attempt at organization after an initial plan has already been established. In many cases an embryo of from 10 to 12 days of age was formed before the aberrations on the head began to be noticeable. It is therefore concluded that, as differentiation proceeds, certain cells of the head region retain the ability (for a few days) of producing new embryonic organization. Ordinarily these regulative cells remain dormant until they no longer have the power of independent differentiation, but irradiation arouses them to new activity. These regulative areas are probably not stimulated directly by the radiation. The dosages necessary to produce the aberrations of Type 2 are certainly not stimulative to the whole embryo as marked injury usually results. Cells of high developmental potentiality are generally regarded as being most sensitive to radiation (see Dugger, pp. 405–6). Indeed, the only available reason for the destruction of the more differentiated cells and the apparent resistance of the regulative or aberrant cells is that

the former may be the more developmentally active during and immediately following the irradiation. The most plausible explanation at the present is that highly active cells which normally control the development of the regulative ones are injured by the irradiation, and the regenerative cells then begin to exert their differentiating power until they again come under the influence of other regions.

The fact that the aberrant outgrowths apparently originate from the head region and not from the thorax might lead one to believe that the differentiation center in *Melanoplus* is not in the same region as it is in the Odonata, Hymenoptera, and in *T. asynamorus*. It must be remembered, however, that gross Roentgen-radiation effects usually appear only after a latent period, which is quite long if the dosage is not heavy. It is possible that, with the posterior progress of differentiation and growth, some cells of the original differentiation center are retained in the head region and have become separated from the thoracic region before their renewed activity begins.[2]

CONCLUSIONS

1. The frequency of appearance of abnormal embryos of *M. differentialis* is highest on the sixth and seventh days of development at 25° C. This is a period of extensive and fundamental differentiation.

2. There is an optimum dosage of from 200 to 400 roentgens for the production of abnormal embryos.

3. The size and character of the different abnormalities apparently indicate that the developmental potentiality is more limited at the posterior regions.

4. There are apparently two general classes of abnormalities: (1) local aberrations such as duplications or bifurcations of structures pertaining to that particular region and (2) secondary outgrowths which are apparently attempts at the formation of 1 or more new embryos.

5. The anomalies are probably the result of the selective action of Roentgen radiation on more active cells. The destruction or injury of certain cells probably results in (1) splitting of anlage and (2) removal of inhibitory control over the development of cells of the head regions which apparently have retained the potentiality of forming whole embryos.

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[2] Since the preparation of this manuscript Dr. E. E. Carothers (1936) has investigated the cytology of these abnormal growths. It has been indicated in a preliminary report that the structural anomalies apparently develop from groups of normal cells that have been isolated by lesions resulting from the X-radiation.

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MUTATIONS IN PARTHENOGENETIC CLADOCERA INDUCED BY X-RADIATION[1]

(One plate)

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MUTATIONS have been known to appear among Cladocera under normal laboratory conditions without subjecting them to any treatment and when bred only parthenogenetically (Banta, 1916; Banta and Wood, 1928*b*). In this paper are reported mutations induced by X-radiation in one species of parthenogenetic Cladocera. The method of production is described; and, also, the probable significance of the structural and physiological manifestations is discussed.

EXPERIMENTAL

Two types of X-ray machines were employed in this work. Successful results in altering the germinal plasm of the individuals subjected to experimentation were obtained with each of these machines.

The first X-ray apparatus consisted of a Müller water-cooled hot-cathode type tube, having a tungsten target. The potential was supplied by a three-phase constant-potential Kenotron rectified type power unit. The X-rays were filtered through a 1-mm. aluminum screen, producing an effective wave-length of 0.360 Ångstrom units and delivering, under these conditions, 193.8 roentgens per minute. An irradiation under these conditions consisting of about 10,000 roentgens was found to be effective in altering the germ cells of the individuals and leading to the production of structural and physiological changes.

The second apparatus consisted of a molybdenum target X-ray tube operated at 30 peak kilovolts on a single-phase half-wave rectified current power plant. The greatest portion of the energy under these conditions was emitted as relatively soft X-rays rich in molybdenum Kα radiation which has an average wave-length of approximately 0.712 Ångstrom units. The tube was water cooled, and an aluminum filter 0.05 mm. in thickness was employed. This represents the thickness of the small aluminum basket in which the animals were placed with a few drops of the culture medium. Under these conditions an irradiation consisting of about 25,000 roentgens was found to be effective in altering the germinal plasm of the individuals.

The animals employed for the experimental study were *Simocephalus vetulus*, a species reared parthenogenetically in the laboratory for several years before being utilized for the experiments. All of the animals were derived from a single clone, i.e., all were descendants by parthenogenesis from one female brought into the laboratory from a pond in the vicinity of Poughkeepsie, New York. The method of rearing the animals and the methods employed in X-radiation are the same as previously reported (Obreshkove and King, 1932; Obreshkove, 1934).

[1] The authors are indebted to the Chemical Foundation, Incorporated, New York City, and to the Hendricks Research Fund (Syracuse University, College of Medicine) for placing at their disposal the equipment necessary to the progress of this investigation.

In producing mutations of the type described in this paper with a strength of X-radiation which was known to be effective, it was found that success was dependent upon choosing of animals for irradiation at a precise stage in the development of the germ cells. The animals were subjected to experimentation late in the third instar, or about 2 hours before the eggs from the ovary were deposited in the brood pouch. Failure to identify accurately this stage in the development of the germ cells led to negative results with a strength of X-radiation which has been definitely established as being effective. These negative results led us to the conclusion that the critical period of the germ cells, at which they are most susceptible to X-radiation for the production of mutations, requires still more accurate localization than heretofore we have been able to state. Banta and Brown (1929) have identified the period for the control of male production in *Moina macrocopa* as being about four hours before the deposition of eggs in the brood pouch.

The changes which have been produced by X-raying the ovaries of the individuals described in this paper have reference to two structures: (1) the terminal claws of the heel of the postabdomen, and (2) the antennae. The morphological changes in these two structures have been observed in the same individual sometimes singly, sometimes together. The changes in the terminal claws were permanent in character; i.e., if they were found to be present in the young immediately after their release from the brood chamber of the mother, they were retained throughout the life of the individual. The changes observed in the antennae of the animals, on the other hand, were temporary in character. They were in evidence when the young were in their first instar, but immediately after the first ecdysis the abnormalities disappeared. These observations are of considerable interest, particularly when correlated with other known facts pertaining to other mutations in Cladocera appearing under normal laboratory conditions without their having been subjected to any treatment. They will be considered in more detail later in the paper.

The normal structural features of a *S. vetulus* are shown in Figure 1. The animal shown here is one day old and was obtained from the normal stock. At the extreme posterior portion of the body of the animal is shown the postabdomen. It is heel-like in appearance, bearing at its extreme free end two prominent elongated structures, the terminal claws. Posterior to each terminal claw of the heel, there follow about ten anal spines, each succeeding spine gradually decreasing in length. The postabdomen is flexible and is capable of being bent forward and backward. The postabdomen and the antennae constitute powerful organs for the execution of the swimming movements. It is important to note that in the normal animal the terminal claws are unsegmented.

Figure 2 shows a *S. vetulus* of the same age as that shown in Figure 1. This animal was obtained from the mutant clone. This clone was started from a single individual, the progeny of a mother whose germ plasm had been altered previously by X-radiation. The structural organization of the postabdominal terminal claws is now seen to be in marked contrast with that shown in Figure 1. A constriction is observed to appear precisely in the mid-region of each claw. Such constrictions, dividing each claw into two well-defined segments, were observed to be similar in position in every individual in which they were found to be present. They are not to be confused with the distortions produced in the terminal claws, and in other appendages, by the direct dehydrative influence of the X-rays on animals.

The changes in the terminal claws shown in Figure 2 were produced in our more recent

experimental work. Similar results also were obtained four years earlier. The early observations, however, because of other laboratory routine at the time, were not carried beyond several generations after each successful production of the mutant structure. In our present study, only one line was isolated (line 6); and the individuals in each newly released brood of young in this line were followed in a more systematic manner. This line represents at present over fifty generations covering about one year of observations. The line traces its history to a single individual which possessed the mutant character. Line 6 continues to produce individuals which show the mutant characteristic, as well as animals which are normal in appearance. In a single clutch of young there may be found both types of individuals, or the mutant character may be entirely absent. But even individuals which appear to be normal possess the potentiality to produce in subsequent generations the two types of young, namely, individuals which are normal in appearance and those which possess segmental claws. In this respect our observations are not unlike those of Banta (1916) for a strain of a sex intergrade stock produced by mutation.

The segmental condition of the terminal claws shown in Figure 2 appears more prominently immediately after the shedding of the carapace. At this time the external covering of the postabdominal claws is more transparent and more flexible. It represents at this stage in its development the outer portion of the skin of the animal. As the outer covering differentiates further after each ecdysis, it becomes more difficult to observe the segmental condition of the terminal claws. In fact, at times the segmental condition appears to be entirely absent. Immediately after the next molt, however, the mutant character again becomes evident.

In the normal animal the function of the postabdominal claws is to comb (by their constant forward and backward motion) the legs, which are respiratory in function. This combing renders the legs free from foreign matter and particularly from certain protozoa which tend to cling to them. It appears that an overaccumulation of microörganisms or other foreign substances on the legs interferes with the normal functioning of these structures. This finds support in the fact that the segmental claws of the postabdomen are often useless to the animal, for with each combing movement the distal portion of each claw bends, and has often been observed to break. For this reason animals with segmental claws often do not survive to reach sexual maturity, particularly if the segmental claws have not been sufficiently reinforced in the process of forming a new covering after each molt.

The variation in structural organization of the postabdominal terminal claws described in this paper is of additional interest, for it furnishes us also with a very fine example of the role which a structure modified through mutation may play in the struggle for existence of the organism. This role can be followed in the laboratory in a comparatively short period of time. The rearing of animals of the mutant stock for a long period, without an endeavor to select for the mutant structure, in time reduces considerably the appearance of the mutant character among the progenies.

MINIATURE ANTENNAE

The variation in structural organization of *S. vetulus*, heretofore described, pertaining to the terminal claws produced originally by X-rayed germ cells, has often, in the course of the study, been observed to be accompanied by another type of structural variation

involving the antennae. Because of the smaller size of these structures under such conditions, they are referred to here as "miniature antennae."

In *S. vetulus* each antenna arises from a comparatively broad basal appendage, which at its distal end becomes biramus in nature. Two segmental lateral structures follow from the basal portion. Each lateral appendage consists of three prominent segments. From regions where two segments come in contact with each other there are given off, in the normal animal, prominent setae, which in the young, immediately after their release from the brood chamber of the mother, are of considerable length, often longer than the antennae themselves. These setae, as well as other structural features of the antennae, are fully formed and are easily visible in the young, even before their release.

Figure 3 shows the structural features of the antennae of a normal *S. vetulus*. The animal was one day old and was obtained from the normal stock. Figure 4 shows, on the other hand, the structural features of an animal of the same age as that shown in Figure 3, obtained from the mutant clone. This animal shows certain modifications in the structural organization of the antennae. It is evident, from an inspection of these two figures, that the antennae of the individual which has undergone modification are shorter and somewhat broader and are distinctly characterized by the absence of prominent setae. The animal shown in Figure 4 does not represent a first-generation young produced immediately after the X-raying of an ovary, but represents an animal many generations later, descendent from the original mutant from which the line 6 was started.

In a clutch of young which has been released from the brood chamber of a mother of the mutant line, there may be found young which are normal in appearance and individuals which show variations either with regard to the structural features of the antennae or the terminal claws, or variations with regard to both of these features in the same individual. A normal individual in the mutant stock possesses the potentiality to produce, in subsequent generations, both normal and abnormal young. Often an entire clutch of young was found to be abnormal with regard to the structural features of the antennae. Also, several generations of young may appear to be entirely free from any variations in structural organization, and then suddenly, in subsequent generations, the abnormalities reappear.

The miniature antennae differ from the segmental postabdominal condition in that, whereas the latter remained unchanged with subsequent growth and differentiation after its appearance, the former showed structural instability. In every case of miniature antennae the structures revert to the normal state immediately after the first ecdysis. The reversion occurred with rapidity, suggesting very clearly a sudden release of dynamic forces. These forces necessary for the normal growth and differentiation were apparently held in check until that particular moment. In this respect the condition of the antennae and their subsequent behavior may be looked upon as representing a type of physiological mutation in contrast with the structural mutation involving the terminal claws.

DISCUSSION

From the data presented in this paper it is evident that mutations may be induced in *S. vetulus* by subjecting the ovaries of the animals to an intensity of X-radiation known to be effective for this species at a critical stage in the development of the germ cells. Once the germinal plasm of an individual is altered, leading to the production of structural and physiological variations, the altered state is retained in some of the subsequent generations. It was observed, however, that not all of the individuals in any one single

brood of young in the mutant strain exhibited the mutant character. More generally there were recorded some animals which were apparently normal in structural appearances in every respect and others which exhibited the mutant characters. In this respect the observations recorded here are not different from those of Banta (1916) pertaining to a sex-intergrade stock which appeared under laboratory conditions by a mutation. Banta and Wood (1928*a*) have demonstrated in *Daphnia longispina* the occurrence of recessive mutations in parthenogenesis, under laboratory conditions, which failed to reveal themselves until rendered homozygous, by recombination in sexual reproduction. The dynamic forces in *S. vetulus* which lead to the production of mutations from apparently normal individuals in a mutant stock without the necessity of crossbreeding are of interest. In the absence of more definite data, we are forced to assume that in *S. vetulus* the disappearance and the appearance of the mutant characters in parthenogenesis may be due to a temporary suppression or to a strengthening of the genetic factors responsible for the production of mutations. In another parthenogenetic group, such as the aphids (Morgan, 1928), it is well known that parthenogenetic eggs for several generations may produce only females. Later, however, males and females may be produced parthenogenetically in the same generation. It has been found that under certain conditions the germ cells of these animals extrude an X-chromosome, thus giving rise to the appearance of males.

Our work reveals also that mutations have a physiological effect on the animals. In an earlier paper (Obreshkove and Banta, 1930) it was demonstrated that in a mutant clone of *S. exspinosus*, with regard to the degree of sexuality, individuals which were normal in appearance, but possessing the potentiality to produce, in subsequent generations, sex-intergrade individuals, revealed physiological states which were distinctly different from the individuals in the normal clone from which they were originally derived by mutations. This was demonstrated by a study of the gaseous metabolism of the individuals. In *S. vetulus* the effect of mutations on the physiological state of the organism appears to be expressed, in the absence of other data, by the appearance of miniature antennae and by the subsequent behavior of these structures in the later growth of the individuals possessing them. In view of these observations the thermal race reported by Banta and Wood (1928*b*), as well as their "dwarfs" and "slow developers," becomes of greater interest to us with regard to the possibility which potential genetic factors may play in altering the physiology of these organisms.

SUMMARY

1. The germinal plasm of parthenogenetic *S. vetulus* females was altered by X-radiation leading to the production of mutations.

2. Two types of mutant characters were identified. One involved the structural organization of the terminal postabdominal claws; and the other was temporary in character and physiological in nature.

3. The critical period at which the germ cells appear to be affected by X-rays of known intensity seems to be about two hours before the eggs are laid. Many negative results with an intensity of X-radiation which was found previously to be effective lead to the conclusion that there is need of identifying this period more precisely.

4. In a clutch of young from the same mother of the mutant stock there are found individuals of normal appearance and animals which show the mutant characters, or the mutant characters may be entirely absent.

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PLATE I

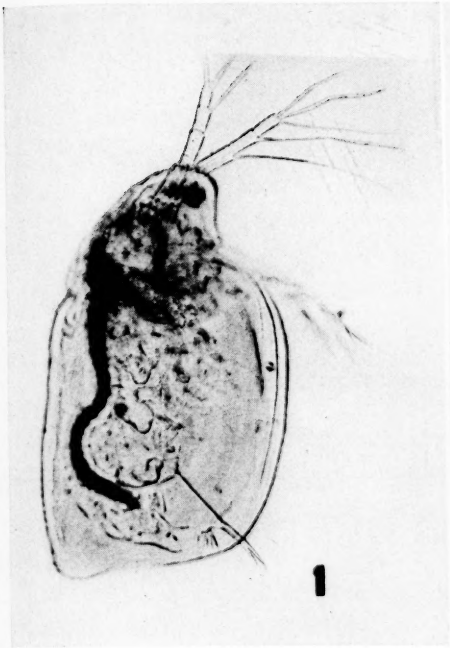
FIG. 1.—*Simocephalus vetulus*, one day old, obtained from normal stock, showing the normal unsegmented condition of the terminal claws of the postabdomen.

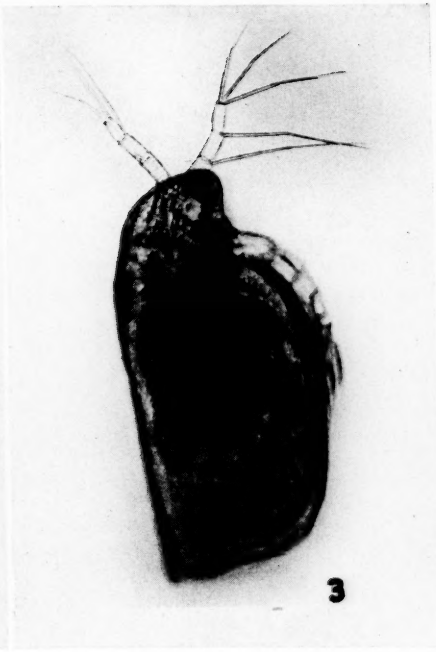
FIG. 2.—*S. vetulus* young of the same age as that shown in Figure 1, obtained from the mutant clone. The terminal postabdominal claws are seen to be segmented, a constriction appearing precisely in the mid-region of each claw.

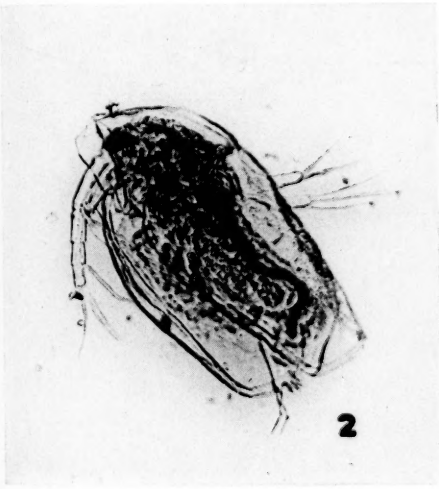
FIG. 3.—*S. vetulus*, one day old, obtained from normal stock, showing the normal structural organization of the antennae.

FIG. 4.—*S. vetulus* young of the same age as that shown in Figure 3, obtained from the mutant clone. The antennae are seen to be characterized by the absence of prominent antennal setae.

PLATE I







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5. The apparently normal individuals in the mutant stock possess the potentiality, in subsequent generations, to produce mutant individuals.

6. The appearance of mutant features in an individual without sexual segregation, and also their temporary absence through several generations, is of special interest.

7. The appearance and the subsequent behavior of miniature antennae reveal that a genetic alteration of the type described here leads to a change in the physiological state of the individuals.

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SOME EFFECTS OF STRYCHNINE ON RECONSTITUTION IN EUPLANARIA DOROTOCEPHALA[1]

(Twenty-one figures)

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IN the reconstitution of pieces of *Euplanaria dorotocephala* head-forms ranging from normal through a graded series of differentially inhibited heads to complete acephaly develop. The frequency of the various head-forms in a given lot of pieces has been called "head frequency". It has been shown that head frequency varies in definite ways with length of piece, level of body from which it was taken, and physiological condition of the animal and that it can be modified by the use of various chemical and physical agents (Child, 1911*a*, 1911*b*, 1916; Behre, 1918; Buchanan, 1922; Hinrichs, 1924; Child and Watanabe, 1935; Rulon, 1936). Most agents used, however, have been depressants, and it was thought that additional evidence as to the factors involved in reconstitution might be obtained by the use of an agent which is known to have a very great stimulating effect on the nervous system of higher animals, namely, strychnine.

The effects of strychnine have been reported for species from the majority of the invertebrate phyla and for many vertebrates. Sherrington (1905), working on the frog, was the first to point out that in strychnine tetanus both muscles of an antagonistic pair contract simultaneously, and the inhibition which occurs in normal reflex movements is absent. The resulting position of the animal depends on the relative strength and leverage of the muscles. He showed that this change of inhibition into excitation is due to the action of the strychnine on the spinal cord. Cushney (1919) observed that strychnine lowers the threshold of response to stimuli and that the tetanus which is induced may be compared to the "start" reflex which normally occurs in response to unexpected sound or touch. Studies by the Lapiques (1913), Bremer and Rylant (1925), Cowan and Ing (1935), and Knoefel (1936) show that strychnine can decrease and completely block conduction in peripheral nerve fibers. The evidence indicates that this peripheral action is depression rather than stimulation.

Strychnine acts specifically to a high degree on the nervous system in the higher invertebrates and vertebrates and when applied centrally results in marked stimulation. However, with animals that have a primitive nervous system or no definite nervous organs, the effect is not distinguishable from that of an anesthetic (various Protozoa—Kritz, 1924; Swindle and Kritz, 1924; and certain coelenterates—Moore, 1917; Child, 1926; and unpublished data of my own on *Tubularia*). Kymograph records demonstrating stimulation after applying strychnine to the ganglia have been made on the octopus (Baglioni, 1905) and on *Limulus* heart (Carlson, 1906). Crozier and Pilz (1924) produced central nervous excitation in grasshoppers and caterpillars by injection and direct application to the thoracic ganglia. Observations of increased activity, loss of co-ordination, and simultaneous excitation of antagonistic muscles have been recorded for a cteno-

[1] The writer wishes to express her appreciation to Dr. C. M. Child, under whom this work was carried on, for his very helpful suggestions and criticisms.

phore (Moore, 1936), a nemertean (Crozier, 1926), annelids (Knowlton and Moore, 1917; Crozier, 1922, 1926), molluscs (Moore, 1916, 1917; Crozier and Arey, 1919*a*, 1919*b*; Crozier, 1920), echinoderms (Pearse, 1909; Moore, 1916, 1918, 1920), and arthropods (Moore, 1913, 1916; Crozier, 1922; Blume, 1930; Clark and Wolf, 1932).

Very little is known of the nervous physiology of flatworms. Moore (1918) observed that in strychninized *Bdelloura* mechanical stimulation caused extension and activity instead of the normal reaction of contraction and adhesion to the substratum. Van Cleave (1929) was unable to alter head frequency in *Stenostomum leucops* with a number of chemical agents including strychnine sulphate although he did find higher head frequency in long pieces, freshly collected specimens, and at low temperatures.

Child (1914*a*, 1914*b*, 1916) and Child and Watanabe (1935) have shown that the degree of development of the head on a cut piece of *E. dorotocephala* depends on two factors: (1) the activity of the cells at the anterior cut surface and (2) an inhibiting factor arising from the posterior cut surface. In short pieces and at posterior levels of the anterior zooid the inhibiting factor is dominant, and consequently head frequency is low. In longer pieces and at anterior levels this factor is less effective. Observations of susceptibility (Child, 1914*a*), carbon dioxide production (Robbins and Child, 1920; Watanabe and Child, 1933), and oxygen consumption (Hyman, 1919*a*, 1919*b*, 1923) have shown that stimulation of the piece as a whole is greatly increased by cutting and decreases progressively with time after sectioning. However, head-frequency determinations on pieces in which the anterior cut was made at varying intervals after the posterior show that some inhibitory influence is present for as long as 3 or 4 days (Child and Watanabe, 1935). Since the stimulation caused by cutting as demonstrated by oxygen consumption and carbon dioxide production disappears in a few hours, it seems probable that the wound healing and early stages of tail regeneration are also concerned with the inhibition. Buchanan (1922, 1923) has suggested that this factor is mainly nervous in character and may be considered to arise from the severing of the nerve cords, and recent experiments by Child and Watanabe (1935) on *E. dorotocephala* and by Watanabe (1935*b*) on *E. tigrina* (= *E. maculata*)[2] indicate that this is probably the case.

With these facts in mind it was thought that investigation of the action of strychnine might contribute additional evidence upon this point. If the strychnine acts on *Euplanaria* as on higher animals, as a specific nervous stimulant, it might conceivably be expected to increase the effectiveness of the inhibiting factor and thus decrease the head frequency. Data from various lines of attack upon this problem will be presented, and their significance discussed.

MATERIAL AND METHODS

GENERAL PROCEDURE

Individuals of the species *E. dorotocephala* which were collected from spring-fed streams near Cary and Rockford, Illinois, and Valparaiso, Indiana, were used for these experiments. In order that they might be in as nearly as possible the same physiological condition they were kept in the laboratory for at least 2 weeks before use. Laboratory care consisted in feeding beef liver two or three times a week, changing the well water in which they were kept daily or on alternate days, and cleaning the pans of accumulated slime at frequent intervals. From a stock which had received at least 1 feeding in the

[2] Revision by Kenk (1935).

laboratory and then had been starved for 10 days to 2 weeks, worms of the same length and width which had not recently undergone fission were selected for use and were cut in pieces with a small knife. Cuts were made in water, and the pieces were transferred immediately into the solution. Cutting the worms in the solution was attempted, but it was found that they became so much more active that cutting pieces of uniform size was very difficult. Pieces from the same body-level were kept in lots of 20 or 25 in 200 cc. of fresh strychnine sulphate solution, made up in well water in finger bowls which were covered and kept in the dark. Concentrations used ranged from M/10,000 to M/500,000. Controls from the same lot of worms were cut and kept in well water under the same conditions as the treated series. Temperature was kept at about 18°–20° C. by thermostatic control of room temperature in winter and by keeping the finger bowls in covered pans surrounded by running water in summer. Exact temperatures were recorded daily in order to make sure that the fluctuations were not great. Observations were made after 10 days to 2 weeks and records made of head-forms; tailless, short, and normal tails; and types of bipolar forms. The head-forms which were described by Child (1911*b*, 1921, 1924) include: (1) normal (Fig. 1), (2) teratophthalmic, with the eyes connected or approximated and the head shape normal, (3) teratomorphic, with a single eye and the cephalic lobes pointing forward or fused in the mid-line, (4) anophthalmic, with an outgrowth of tissue but no eyes, and (5) acephalic, in which the anterior end healed but produced no outgrowth.

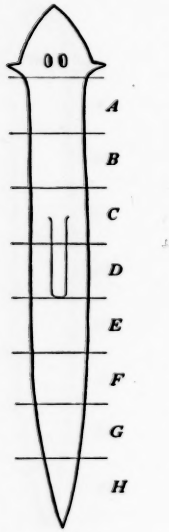


FIG. 1.—Body-levels of pieces used in head-frequency experiments. Each piece is 1/8 of post-cephalic length.

CALCULATION OF HEAD-FREQUENCY INDICES

In all cases only the living pieces were considered in making the graphs, and experiments containing a large number of deaths were discarded. The few experiments in which this was necessary were known to have been affected by rise in temperature, lethal concentration of the agent, or poor condition of the stock.

To obtain numerical values which could be expressed graphically as indices of head frequency the following formula was used (modified from Watanabe, 1935):

$$\frac{100n_5 + 80n_4 + 60n_3 + 40n_2 + 20n_1}{N}.$$

In this formula n_5–n_1 represent the numbers of pieces which develop normal, teratophthalmic, teratomorphic, anophthalmic, and acephalic forms, respectively, and N is the total number of pieces in the series. The coefficients 100, 80, 60, 40, and 20 are arbitrary values assigned to the various head-forms. This formula differs from that given by Watanabe only in that he includes a sixth term in the numerator, $0n_0$, to represent dead pieces. The evidence from the experiments here reported is that deaths often occurred after the heads were differentiated and that many, at least, of the pieces which died were not ace-

phalic but had normal heads. Consequently, dead pieces were omitted from the calculations of head frequency rather than being assigned a value (0) which implies that death was caused by a still greater degree of inhibition than that causing acephalics (value 20). Watanabe's formula, with this slight modification, was used in order that the indices should be comparable to those obtained by other workers in this laboratory. The omission of dead pieces does not greatly alter the values obtained since the frequencies of deaths were low in most cases. The distribution of deaths is discussed later. The scale used, then, ranges from 20 as a base line to represent acephalic forms to 100 for normal heads. In any case the values are purely arbitrary since the frequencies of the various head-forms in control lots show that the groups are not of equal weight. However, this type of graphing is believed to be adequate for comparative purposes.

In the majority of the experimental series bipolar forms appeared. For the purposes of graphing, all the bipolars at each body-level of a given series were combined, irrespective of the degree of development of the heads, and were expressed as percentages of the total number of living pieces at that level. In these groups pieces were also found which behaved like bipolars but had no posterior outgrowth or a short outgrowth which was classed as a short tail. Some of these were probably actually bipolar forms with acephalic or anophthalmic posterior ends. In the later experiments the direction of beat of the cilia on the ventral surface was observed by means of a carbon suspension as a test of the polarity of these forms. However, since this was not done in the early series, and in order to avoid any possibility of over-weighting the bipolar groups, these pieces were recorded as questionable, and only those which were definitely identified as bipolar bythe presence of two heads were included in making the graphs.

RATE OF EYE FORMATION

In order to determine whether or not the solutions were acting on the cells at the anterior cut surface, the time of appearance of eyespots was observed as an index of the rate of differentiation. Pieces were cut three-eighths of the postcephalic length with anterior cut ends at four levels of the anterior zooid. Thus, pieces used included *ABC*, *BCD*, *CDE*, and *DEF* (Fig. 1). Long pieces were used in order that all should develop normal heads. Watanabe (1935*a*) has shown that inhibited heads develop more slowly than normal and that pieces of this size are sufficiently long to produce 100 per cent normals. In five series three lots of 20 pieces each at each level were treated as follows: one lot in M/100,000 strychnine sulphate for 16 hours, another in M/450,000 throughout the experiment, and the third as control in well water, making a total of 100 pieces in each solution at each level. *ABC* and *DEF* pieces in the same solution were cut from the same animals, whereas *BCD* and *CDE* pieces were of necessity from different animals but from the same stock. Only four levels were used because of the fact that in more posterior pieces the eyes develop in the old tissue and cannot be distinguished accurately from body pigment in the early stages. Also, with only four levels, each series contained 240 pieces which was the maximum number that could be observed carefully. Temperatures were controlled as carefully as possible so that the five series would be comparable. Frequent readings showed that the fluctuations were not great and that, except for Series 3, the ranges were almost identical. The extremes of each group were as follows: Series 1, 18.5°–20°; Series 2, 18°–21°; Series 3, 20.5°–22.5°; Series 4, 18°–20.5°; and Series 5, 18.5°–21.5°. C.

Observations were made at 8-hour intervals, and numbers of pieces possessing eyes

were recorded. With the aid of a compound microscope with 16-mm. objective and 10× ocular, the eyespots were discernible as small pigment spots near the boundary of old and new tissue just beneath the ectoderm. By careful focusing and with the aid of strong transmitted light they could be distinguished from the body pigment by their deeper location.

From these data calculations were made of arithmetical means and standard deviations of each lot and of average standard deviations within lots. The statistical probability (P) that as great a difference might be produced by random sampling was calculated with the use of "Student's" formula ("Student," 1925). P was calculated for differences between strychnine-treated and control lots at each level and between the *ABC* and *DEF* pieces in each solution. Percentage values for developmental rates were obtained by taking the mean time of the *ABC* pieces in water as a standard and expressing all the other means relative to it.

EXPERIMENTAL DATA

GENERAL OBSERVATIONS

Intact worms put into strychnine solution (M/1,000–M/500,000) show greatly increased activity. This is at first manifested in both low and high concentrations by elongation of the head and of the body, lifting of the head from the substratum and waving it back and forth, rapid movement, and muscular tremors. Such worms will not feed, rheotaxis is not exhibited, and when turned over, their righting reaction is either very slow or absent. They are very easily stimulated to move by sudden increase in illumination or by slight agitation of the solution, but their locomotion seems poorly co-ordinated and they are easily dislodged from the substratum. Although they are highly motile, they do not show the tetanus, caused by touch or jarring, characteristic of strychnine action on higher forms. The typical reaction of untreated *Euplanaria* to jarring of the dish is a shortening of the body and firmer adhesion to the bottom. However, strychninized specimens react instead by elongation and increased motor activity. This is a case of "reversal of inhibition" similar to that described by Moore (1918) for the marine triclad *Bdelloura*. In lethal concentrations (M/1,000–M/200,000) the animals soon exhibit spasmodic contractions, violent coiling and twisting of the body, and inability to remain attached. Small splits appear in the body wall, and disintegration of the anterior zooid proceeds in general in an antero-posterior direction. Disintegration of the posterior zooid usually begins before that of the anterior zooid is complete, but the coiling of the worm makes the course of the disintegration difficult to follow. Muscular tremors are present until the cytolysis is complete.

Cut pieces show the same general effects with regard to elongation, increased activity, muscular contractions, and inability to remain attached. They disintegrate in lower concentrations than whole worms and also at lower temperatures than do the controls. The pieces are often found ventral side up with both ends raised—a position which seems to be a state of tonic contraction with the body bent by the strongest muscles. Split places, in pieces as well as in whole worms, often appear in high concentrations, but if not too extensive, will heal if the pieces are returned to well water. Pieces reconstituting in strychnine appear very elongate with long, narrow, and pointed heads. However, if these are placed into well water for a few hours, they return to normal shape, and the elongation is seen to be functional rather than morphological.

HEAD FREQUENCY

One-eighth pieces of 16-mm. worms.—To determine the effect on head frequency, experimental lots were exposed for varying lengths of time to various molecular concentrations of strychnine sulphate made up in well water. Preliminary experiments to determine barely sublethal concentrations showed that for 16-mm. worms M/450,000 for continuous exposure, M/100,000 for 16 hours, and M/300,000 for 3 days were the most effective. Worms were cut into eighths of the postcephalic length as shown in Figure 1.

The most noticeable effect in all the solutions is a very marked increase in head frequency (Table 1 and Figs. 2, 3, and 4). This increase occurs not only in pieces from the pharyngeal region (*C* and *D*), which also show higher head frequencies in anesthetics, but also in any lots of *A* and *B* pieces which show inhibited heads in the controls. In

TABLE 1

HEAD-FREQUENCY COUNTS IN FOUR LOTS, EACH CONTAINING 25 16-MM. WORMS

Series	Type of Head	A	B	C	D	E	F	G	H
Strychnine, M/450,000	Normal	80	63	38	39	49	48	73	99
	Teratophthalmic	1	21	22	26	23	30	16	.....
	Teratomorphic	.....	1	14	6	2	2	1	.....
	Anophthalmic	.....	.....	13	9	3	6	6	.....
	Acephalic	.....	.....	3	15	14	11	2	.....
	Bipolar	.....	.....	2	2	4	.....	.....	.....
	Dead	19	15	8	3	5	3	2	1
Index		99.8	94.6	78.0	74.2	80.6	80.2	91.0	100
Control	Normal	72	8	.....	.....	4	13	35	99
	Teratophthalmic	23	18	.....	.....	9	20	40	.....
	Teratomorphic	2	9	.....	.....	6	7	9	.....
	Anophthalmic	1	10	2	2	14	17	3	.....
	Acephalic	2	53	98	97	67	40	13	.....
	Bipolar	.....	.....	.....	.....	.....	.....	.....	.....
	Dead	.....	2	.....	1	.....	3	.....	1
Index		92.4	43.3	20.4	20.4	33.8	49.5	76.2	100

such lots head frequency is usually decreased by narcotics. The exact amount of increase in strychnine varies slightly with different lots of worms, but in practically every case the curve for the experimental group is above that for the control. However, in one series the *H* pieces (Fig. 1), which usually produce 100 per cent normals, developed a few teratophthalmics in water and still more in strychnine (Figs. 3 and 4). The curves for the controls vary slightly also in different lots of worms. In experiments in which the controls give rather high head-frequency indices the strychnine-treated series is very much higher.

Since strychnine-treated animals are easily activated by jarring and by sudden exposure to light, and it is known that mechanical stimulation will increase head frequency (Child, 1911*a*, 1920), it was thought that possibly some of the great increase noted in experimental lots might have been due, in part at least, to the agitation involved in changing the solutions and moving the dishes to make observations. Therefore, simultaneously with other series of continuous exposure to M/450,000 strychnine sulphate, which were

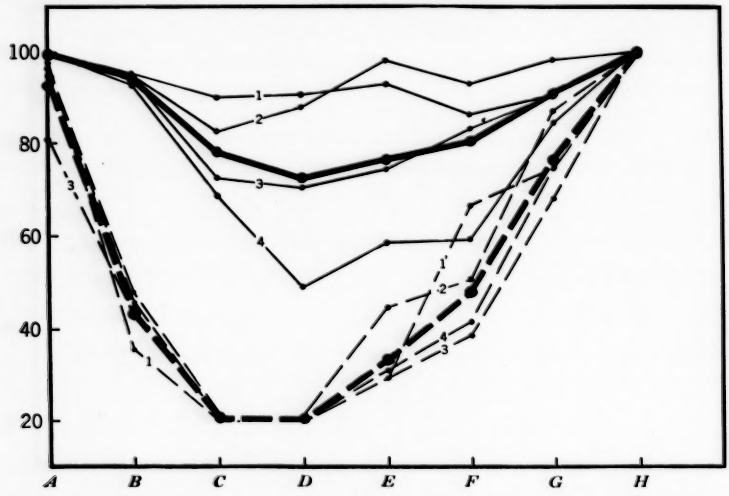


FIG. 2.—Head frequencies of 1/8 pieces of 16-mm. worms reconstituted in M/450,000 strychnine sulphate (continuous lines) and controls (broken lines). Corresponding experimental and control lots are indicated by numbers 1–4. Values obtained from combined counts (from Table 1) are shown by heavy lines. Ordinates are head-frequency indices and abscissae body-levels.

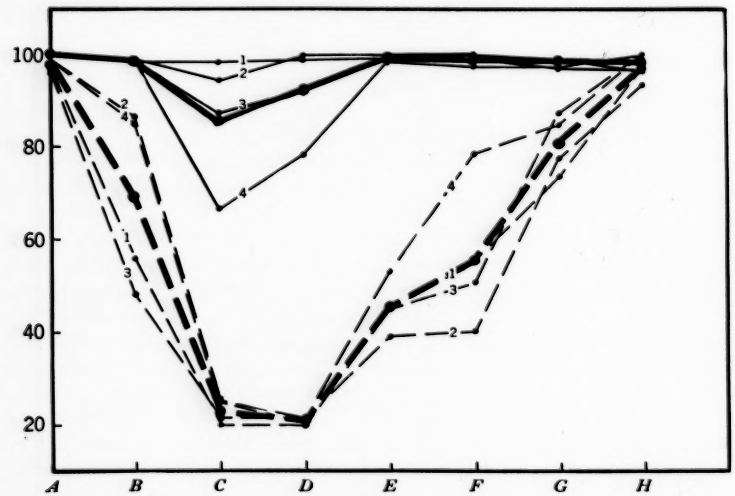


FIG. 3.—Head frequencies of 1/8 pieces of 16-mm. worms reconstituting in water after 3 days' exposure to M/300,000 strychnine sulphate (continuous lines) and controls (broken lines). Corresponding experimental and control lots are indicated by numers 1–4 and combined data by heavy lines. Ordinates are head-frequency indices and abscissae body-levels.

kept in finger bowls and treated in the usual manner, 50 experimental and 50 control worms were cut and placed in 1-liter Erlenmeyer flasks which were stoppered and placed in the dark. After 2 weeks, during which they were neither moved nor exposed to light, the types of heads were counted and head-frequency indices calculated. It was found that the values for both experimental and control lots which were not moved were slightly lower than those which were handled. However, those in strychnine were considerably higher than their corresponding controls, showing that mechanical stimulation played only a small part in the increase.

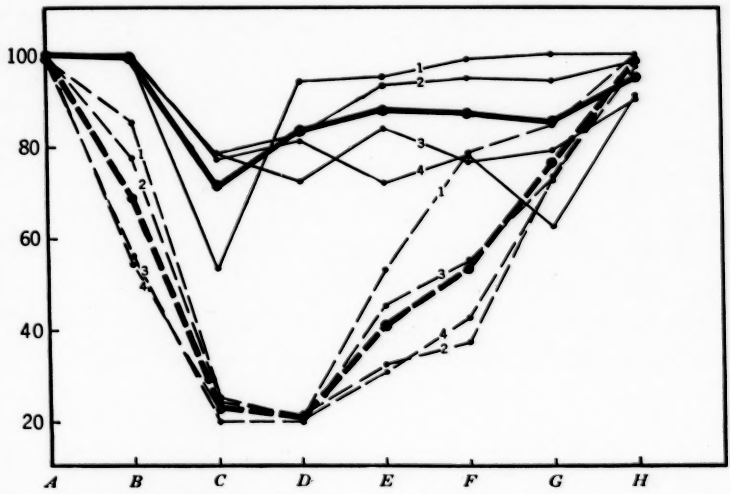


FIG. 4.—Head frequencies of 1/8 pieces of 16-mm. worms reconstituted after 16-hour exposure to M/100,000 strychnine sulphate (continuous lines) and controls (broken lines). Corresponding experimental and control lots are indicated by numbers 1–4 and combined data by heavy lines. Ordinates are head-frequency indices and abscissae body-levels.

In Table 1 are given the numbers of the various head-forms produced by continuous exposure to M/450,000 strychnine sulphate and also the head-frequency indices for each body-level. The latter are graphed in Figure 2. The curves shown in Figures 3 and 4 were obtained from similar lots of data. All three graphs (Figs. 2, 3, and 4) show the curves for each of the four pairs of experimental and control lots in light lines and the curves obtained from the combined data in heavy lines. The curves for corresponding experimental and control lots, which were cut at the same time from the same collection of worms, are designated by the same number.

The pieces exposed to M/300,000 for 3 days show the greatest increase (Fig. 3), although the curves for the treated lots in the other experiments are also conspicuously higher than those of the controls. Pieces exposed to M/100,000 give the most variable results (Fig. 4), although similar in general to those obtained with lower concentrations. Preliminary experiments, using only the anterior zooid of worms from the same collection and exposing the pieces for 8, 12, 18, and 20 hours, gave more uniform results than

repetitions of 16-hour exposures on pieces from different lots of worms even though the latter were kept under laboratory conditions and starved for the same length of time. This indicates that different lots of worms exhibit varying degrees of susceptibility to treatment. The percentage of bipolar forms produced is greatest in high concentrations. This point will be considered in the section on "Regeneration at Posterior Cut Surface."

Delayed posterior section.—It is known that the head frequency of very short pieces increases when the posterior cut is made at increasing intervals after the anterior cut (Child and Watanabe, 1935). A series of experiments to test the effect of such delay in strychnine as compared with the condition in water was made on pieces from the *E* level (Fig. 1). Four lots were run, each consisting of 25 pieces in strychnine and 25 in water

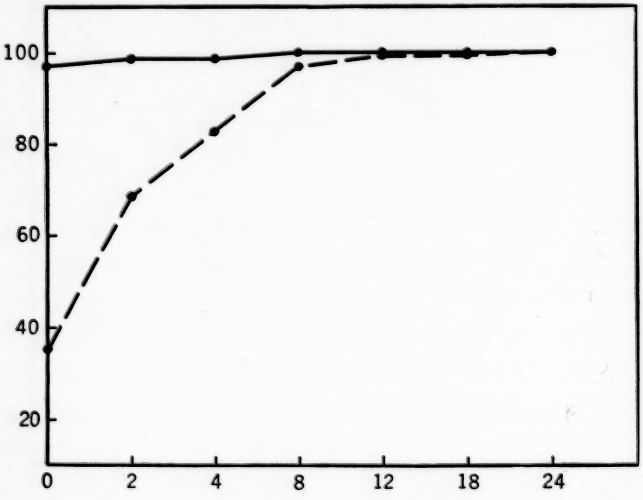


FIG. 5.—Head frequencies in M/500,000 strychnine sulphate (continuous line) and control (broken line) with delay of posterior section. Ordinates are head-frequency indices and abscissae intervals of delay in hours.

for each interval of delay. The first cut was made directly behind the mouth, where head frequency is lowest in controls, and the second cut was made 1 mm. behind the first at intervals of 0, 2, 4, 8, 12, 18, and 24 hours. Worms of 12–14 mm. were used, and the experimental series was exposed to M/500,000 strychnine sulphate. A slightly lower concentration than in the preceding experiments was used because of the fact that the pieces were smaller and from smaller worms and showed a higher susceptibility to the treatment.

Of the treated animals nearly all were normal after 0, 2, and 4 hours' delay and all were normal from 8 hours on; whereas the controls gave very low values at 0 hours, increasing to nearly all normal at 12 and 18 and all normal at 24 hours (Fig. 5). The large numbers of bipolar forms which occurred in these experiments will be described in the section on "Regeneration at Posterior Cut Surface."

Delayed anterior section.—A similar series was run with the anterior cut delayed. Child and Watanabe (1935) have shown that, for attainment of completely normal head fre-

quency, the delay must be very much longer than in the preceding series. The first cut was made a short distance behind the mouth, and the second made 1 mm. anterior to it at intervals of 0, 24, 48, 72, and 96 hours. Experimental lots were placed in M/500,000 strychnine sulphate and controls in well water.

In controls head frequency decreased from 0 to 24 hours and then increased with longer intervals of delay. This corresponds to the results obtained by Child and Watanabe (1935) on *E. dorotocephala* and Watanabe (1935*b*) on *E. tigrina*. In strychnine the results were similar to those for the delayed posterior-section experiment, i.e., high head frequency at all periods (Fig. 6).

REGENERATION AT POSTERIOR CUT SURFACE

In certain experiments large numbers of bipolar and tailless pieces were observed. Consequently, it was thought wise to consider the frequency of such developments at the posterior cut surface under a separate heading.

The numbers of bipolars appearing in 1/8 pieces are expressed as percentages of living pieces at each level in Figure 7. In all the solutions the *E* level showed the highest number, and the percentages corresponded to the concentration of the agent. The largest number (26.4 per cent at the *E* level) appeared in M/100,000, fewer (5.2 per cent) in M/300,000, still fewer (4.2 per cent) in M/450,000, and none in water at any level. Thus the high concentration is very much the most effective in producing bipolars. In Table 2 are recorded the types of outgrowth at the posterior cut surface in lots treated with M/100,000 and in their controls. This shows the distribution of various types of bipolar forms and also of tailless pieces and deaths. The form of the curve for bipolars (Fig. 7) is very regular, with a low peak at the *B* level and a high flat peak at the *E* level. This peak in *B* pieces also showed very distinctly in a few experiments with 1/16 pieces. Factors involved in this distribution will be considered in the discussion.

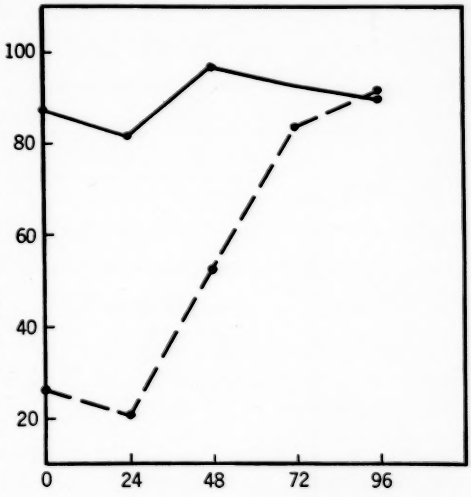


FIG. 6.—Head frequencies in M/500,000 strychnine sulphate (continuous line) and control (broken line) with delay of anterior section. Ordinates are head-frequency indices and abscissae intervals of delay in hours.

When the anterior cut was delayed, very few bipolars appeared. In strychnine several bipolars (18.2 per cent) and tailless (18.2 per cent) appeared after simultaneous sectioning whereas only one taillesss and no bipolars developed in water. After 24 hours' delay a few bipolars were produced in strychnine (8.1 per cent), but with subsequent delay all formed normal tails (Fig. 8).

The largest numbers of bipolar pieces, however, occurred in the experiments on delayed posterior section. With increasing intervals of delay the numbers of bipolars in

water increased from 1.1 per cent of living pieces after simultaneous anterior and posterior section to a maximum of 25.3 per cent after 12 hours' delay and 26.1 per cent after

TABLE 2

REGENERATION AT POSTERIOR CUT IN 1/8 PIECES OF 16-MM. WORMS
COMBINED DATA FROM FOUR LOTS EACH OF 25 WORMS

Series	Type of Outgrowth	A	B	C	D	E	F	G	H
Strychnine, M/100,000	Normal head	.....	5	2	7	6	9	5	.....
	Teratophthalmic	.....	.....	1	3	10	4	2	.....
	Teratomorphic	.....	2	1	5	5	3	4	.....
	Anophthalmic	.....	1	.....	2	2	5	1	.....
	No outgrowth	39	39	25	16	23	25	24	.....
	Short tail	6	15	12	17	12	17	22	.....
	Normal tail	47	28	45	23	29	30	37	94
	Dead	8	10	14	27	13	7	5	6
Control	Normal head	.....	.....	.....	.....	.....	.....	.....	.....
	Teratophthalmic	.....	.....	.....	.....	.....	.....	.....	.....
	Teratomorphic	.....	.....	.....	.....	.....	.....	.....	.....
	Anophthalmic	.....	.....	.....	.....	.....	.....	.....	.....
	No outgrowth	1	.....	.....	.....	4	3	.....	.....
	Short tail	.....	.....	.....	.....	7	.....	5	.....
	Normal tail	92	95	97	96	89	97	94	99
	Dead	7	5	3	4	.....	.....	1	1

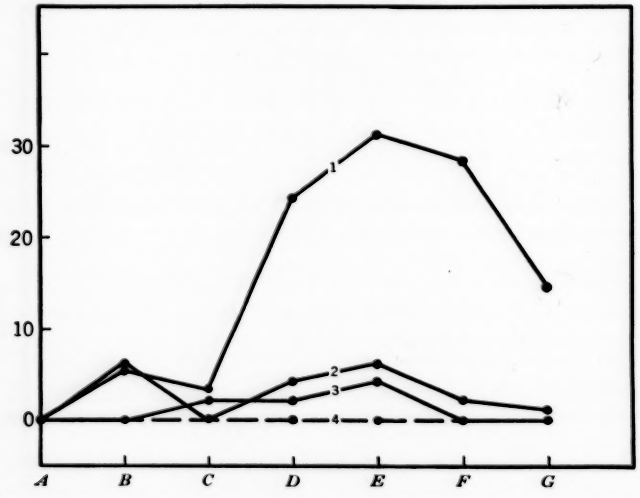


FIG. 7.—Percentages of bipolar forms produced in 1/8 pieces of 16-mm. worms in (1) M/100,000 strychnine sulphate, (2) M/300,000, (3) M/450,000, and (4) well-water control. Ordinates are bipolars in percentage of living pieces and abscissae body-levels.

18 hours (Table 3; Fig. 9). In strychnine large numbers appeared in all groups: 44.2 per cent after simultaneous section, 73.8 per cent after 12 hours' delay, and 38.8 per cent after 24 hours' delay.

All combinations of head-forms were produced (Figs. 10–19), but in most cases at least one head was normal, and the other cut surface produced a normal head more often than any other type. The more nearly normal head was taken as the originally anterior end.

Tailless pieces also occurred in large numbers in the 1/8 pieces exposed to M/100,000 (Table 2) and the delayed posterior-section experiments (Table 3). In the 1/8 pieces from different levels of the body these showed a differential distribution which was in general the reverse of that of the bipolars in the anterior zooid. In *A* pieces, where no bipolars appeared, the tailless were very numerous (42.4 per cent), but in the *D* and *E* pieces, where the bipolars were more numerous (23.3 per cent and 26.4 per cent), fewer tailless were found (21.9 per cent and 26.7 per cent).

Possible explanations for the occurrence of these forms are (1) direct inhibition of the cells at the posterior cut, (2) increased "scale of organization," or (3) incomplete reversal of polarity. In order to test the first hypothesis long pieces consisting of the head, *A*, and *B* were compared with *B* pieces alone in water and in strychnine. This level was used because many tailless pieces occurred here in strychnine experiments. No tailless pieces were formed in water in either long or short pieces, and none in strychnine in the long pieces, but several occurred in strychnine in the short pieces. This indicates that the action was not one of direct inhibition of the cells at the posterior cut surface but was related to the size of the piece and possibly also the presence of the head.

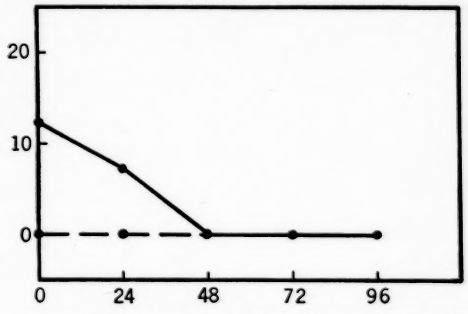


FIG. 8.—Percentages of bipolar forms with delay of anterior section in M/500,000 strychnine sulphate (continuous line) and control (broken line). Ordinates are bipolars in percentage of living pieces and abscissae intervals of delay in hours.

The position of the pharynx has been used as an index of what Child has called "scale of organization." He found (1911*a*, 1912, 1929) that in pieces regenerating in anesthetics the pharynx is smaller and closer to the head than in controls, and that with high temperature the reverse occurs. Observations of the position of the pharynx reconstituting in strychnine showed no significant alteration.

Reversal of polarity in questionable cases was determined by observing the behavior of the regenerated worms and the direction of beat of the cilia on their ventral surfaces. With the aid of a fine carbon suspension under a binocular microscope the direction of the current produced by ciliary action can be seen very easily (Rustia, 1925). In normal worms the cilia beat in an antero-posterior direction down the entire length of the worm, but in bipolar forms they beat from both ends toward the middle (Figs. 16–20).

In many of the pieces with no posterior new tissue the use of a carbon suspension demonstrated reversal of the ciliary beat at the healed surface. Such pieces often developed secondary tail-like lateral outgrowths of old tissue (Fig. 19). From their behavior, appearance, and ciliary reversal these were believed to have been actually bipolars with one completely inhibited head. Other pieces which produced no posterior new tissue, but which also had no ciliary reversal and no lateral outgrowths, were probably cases of inhibited tails (Fig. 20). Because of the difficulty of distinguishing accurately between these two types, and also because the ciliary beat was not observed in the earlier experi-

ments, none of these was included as bipolars in calculating the percentages of bipolar forms produced.

TABLE 3

REGENERATION AT POSTERIOR CUT AFTER VARYING INTERVALS OF DELAY OF POSTERIOR SECTION

Series	Type of Outgrowth	Number of Hours						
		0	2	4	8	12	18	24
Strychnine, M/500,000	Normal head	16	38	43	34	40	29	10
	Teratophthalmic	4	6	.....	1	.....	.....	.....
	Teratomorphic	11	6	3	5	7	7	11
	Anophthalmic	3	2	2	2	1	2	5
	No outgrowth	21	11	14	14	12	14	29
	Short tail	4	3	.....	1	1	2	3
	Normal tail	18	13	7	6	4	9	9
	Dead	23	21	31	37	35	37	33
Control	Normal head	.....	5	8	9	14	11	14
	Teratophthalmic	.....	.....	.....	.....	.....	.....	.....
	Teratomorphic	1	1	.....	1	5	4	3
	Anophthalmic	.....	.....	2	3	1	2	1
	No outgrowth	28	24	17	24	24	15	33
	Short tail	.....	4	2	3	8	8	3
	Normal tail	63	56	52	39	27	25	29
	Dead	8	10	19	21	21	35	17

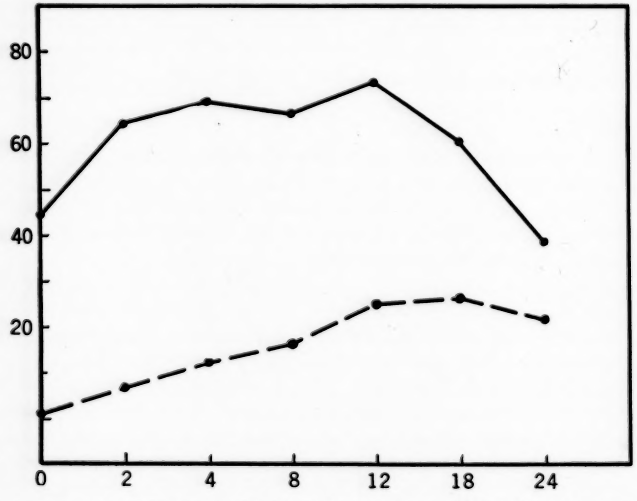
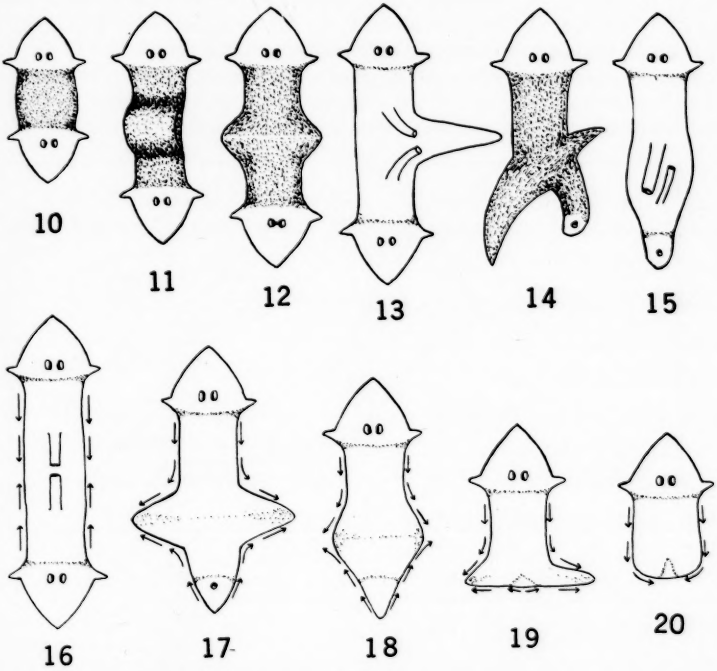


FIG. 9.—Percentages of bipolar forms with delay of posterior section in M/500,000 strychnine sulphate (continuous line) and control (broken line). Ordinates are bipolars in percentage of living pieces and abscissae intervals of delay in hours.

In the bipolar pieces, also, secondary lateral tails were often formed, sometimes on one side and sometimes on both and of varying lengths (Figs. 12–14, 17, 18). In cases in

which one head was normal and one inhibited, these outgrowths were at a greater distance from the normal than from the inhibited head, and one tail was often longer than the other (Fig. 14). A similar situation was observed with respect to the pharynges. In bipolar pieces with unequal heads the pharynges were located at a greater distance from the normal head, and the one determined in relation to the normal head was larger than that of the inhibited head (Fig. 15). Such cases demonstrate very clearly the difference



FIGS. 10–20.—Types of bipolar and tailless forms observed. Figures 12–14, 17–19, lateral tail-like outgrowths of old tissue. Figure 11, dorsal outgrowth of old tissue. Figures 12, 14, 15, 17, and 18, one normal and one inhibited head. Figures 13, 15, and 16, showing pharynges. Figures 16–19, reversal of direction of ciliary beat. Figures 19 and 20, no outgrowth at posterior cut surface.

in scale of organization determined by normal and by inhibited heads. Pharynx development was rather variable; none, one, two, or one with single attached and forked free ends were observed. The majority of the bipolar pieces possessed a dorsal outgrowth of old tissue (Fig. 11). In some this was so large in comparison to the ventral surface that the worms were unable to crawl normally but moved in circles, lying on one side with the two heads almost parallel.

DEATH FREQUENCY

Although the experiments were not planned to show the lethal effects of the agent, small numbers of dead pieces were observed in some solutions because of the fact that the concentrations dealt with were planned to be rarely sublethal, and different lots of

worms varied slightly in their degree of susceptibility. Deaths were highest in the delayed section experiments in which the size of the pieces was small. In the 1/8 pieces the distribution of deaths was seen to be opposite in the two extremes of concentration and exposure periods used (Fig. 21). In continuous exposure to M/450,000, deaths were most numerous in pieces from the anterior end and decreased posteriorly, whereas in 16-hour exposure to M/100,000, deaths increased to a high peak at the *D* level and then decreased. By the end of the 16 hours, when the pieces were returned to water, small split and disintegrating spots could be seen in many *D* and some *C* and *E* pieces. Some of these pieces died later, but the majority recovered.

After 10 days to 2 weeks, when the types of anterior and posterior regeneration were being recorded, pieces were frequently found with disintegrating areas in the old tissue. Occasionally such pieces were a mass of completely disintegrated cells with an intact tail

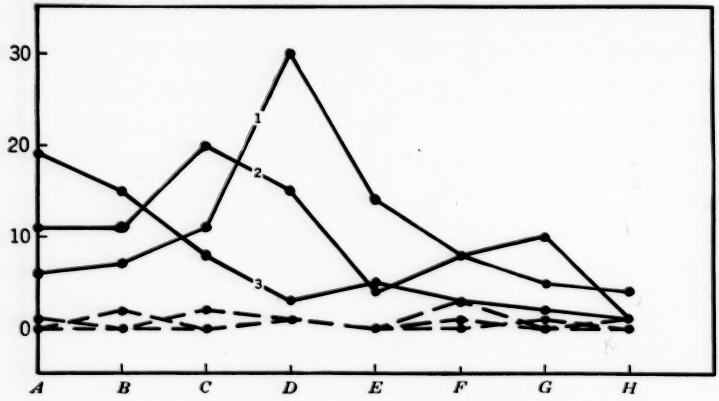


FIG. 21.—Death frequency in (1) M/100,000 strychnine sulphate, (2) M/300,000, (3) M/450,000, and controls (broken line). Ordinates are percentage dead and abscissae body-levels.

and a head in which the eyes were visible. Others were in a still further advanced stage of disintegration in which the type of head was not recognizable. The fact that pieces which had already developed normal heads were seen in various stages of disintegration shows that some, at least, of the pieces which die are not acephalic and suggests that in these experiments deaths ought not to be rated lower than acephalics. Consequently, head-frequency indices in these experiments were computed from living pieces only, and deaths were recorded and figured separately on a percentage basis.

In Figure 21, curves are given for percentages of deaths in the three concentrations used on 1/8 pieces. The deaths in M/300,000 were irregular. However, in continuous exposure to M/450,000 (3, Fig. 21), the largest number of deaths (19 per cent) occurred at the *A* level, and progressively fewer were recorded for more posterior levels. This shows the direct lethal action of this concentration and period of exposure in which the anterior levels are the most susceptible.

In short-time exposure to M/100,000 (1, Fig. 21) the opposite effect was seen. Here deaths were fewest in the *A* pieces (8 per cent) and most in the *D* pieces (27 per cent). This may be attributed to differential recovery in water from the effects of the treat-

ment. That is, the *A* pieces were able to tolerate the solution during the exposure and recover after return to water, but some of the *D* pieces were unable to do so, and consequently the death-rate was higher at the *D* level. Another important factor is the different degree of stimulation following section shown by pieces from various levels. Observations by Child (1914*a*) show that when pieces are placed in potassium cyanide immediately following section the *D* pieces are more susceptible than the *A* pieces. On the other hand, if exposure is made 48 hours after cutting, the reverse is true. Tests of carbon dioxide production (Robbins and Child, 1920) and oxygen consumption (Hyman, 1923) also show that *D* pieces are stimulated by section to a greater extent than *A* pieces. Since all exposures to strychnine were made immediately after cutting, the disintegrating spots in *D* pieces, with temporary exposure to high concentration, and their higher death-rate may have resulted from the greater stimulation of *D* pieces by cutting.

TABLE 4

MEAN TIMES IN HOURS TO APPEARANCE OF EYES IN 3/8 PIECES OF 16-MM. WORMS

Level of Piece	*ABC* (3/8)			*BCD* (3/8)			*CDE* (3/8)			*DEF* (3/8)		
Solutions	M/100,000	H_2O	M/450,000	M/100,000	H_2O	M/450,000	M/100,000	H_2O	M/450,000	M/109,000	H_2O	M/450,000
Mean of means of five series	80.6	77.7	79.8	79.7	78.2	82.2	84.4	81.7	81.6	88.7	83.9	86.1
P	0.0540		0.0330	0.3740		0.0126	0.0540		0.8512	0.0400		0.1036
Average standard deviations within lots	6.6	5.8	6.1	7.1	5.3	5.8	6.6	5.5	4.9	6.0	4.8	6.3
Rates	96.4	100.0	97.4	97.5	99.3	94.5	92.1	95.1	95.2	87.6	92.6	90.2

RATE OF EYE FORMATION

Observations of times of appearance of eyes were made on pieces long enough to produce normal heads as described earlier. In Table 4 are given the means of means and average standard deviations for the five series in each solution. The statistical significance (*P*, Table 4) shows that there is a real though slight delay in anterior pieces with continuous exposure and in posterior pieces with temporary exposure. This difference in reaction to the two solutions resembles the distribution of deaths just described. In both cases the continuous exposure to M/450,000 produced the greatest effect on the anterior pieces, and temporary exposure to M/100,000 showed the reverse situation.

Rates of development (Table 4) are calculated as described in the section on methods by expressing mean times as percentage values relative to the time of the *A* pieces in water. Such rates show a progressive decrease between adjacent levels with increasing distance from the head. This decrease is very regular in water and shows a general, though less regular, trend in the experimental solutions. Comparisons of the *ABC* with the *DEF* pieces in each solution show that the *DEF* pieces are significantly slower and

that the interval is greater in the strychnine solutions than in water. The significance (P) for this difference in M/100,000 is 0.0126; in M/450,000, 0.0148; and in water, 0.0300. The standard deviations for each lot are slightly smaller than those given by Watanabe (1935*a*) since observations were made at 8-hour intervals instead of at the 12-hour intervals which he used. The increase in variability from anterior to posterior levels which he reports was not found.

FISSION

Four experimental and four control lots of 50 12–14-mm. worms with heads removed were placed in 1 liter each of M/300,000 strychnine sulphate or well water. Two experimental and two control lots were kept in earthenware containers in order to determine whether slight irregularities of surface would tend to induce fission, and the other four were kept in glass crystallization dishes. All were covered and placed in darkness. Of the 200 worms in earthenware dishes 52 per cent in water and 2 per cent in strychnine underwent fission. In the glass dishes fissions occurred in 56 per cent in water and none in strychnine, making a total of 54 per cent fission in controls contrasted with only 1 per cent in the test solution. Instead of being increased, the number of fissions in controls in earthenware dishes is actually lower than in the glass dishes, but the difference is not significant. However, the very low number in experimental lots is striking evidence that strychnine inhibits fission even in headless animals.

DISCUSSION

Observations on the reactions of *E. dorotocephala* to strychnine treatment indicate that in this form the effects are intermediate between the depression exhibited by Protozoa and Coelenterata and the high degree of stimulation in the higher invertebrates and the vertebrates. Planarians in strychnine show the typical "reversal of inhibition" of antagonistic muscles, increased susceptibility to a rise in temperature, and greatly increased motor activity especially after touch, jarring, or exposure to light. However, they show no sudden contractions in response to such stimuli and no indication of the tetanus characteristic of strychnine action on higher forms. The increased activity is accompanied by loss of co-ordination and inability to adhere to the substratum similar to that observed by Hyman (1919*a*) on pieces in KCN and by Rulon (1936) in high concentrations of carbon dioxide. However, the incidence of fission differs greatly. In carbon dioxide (Rulon, 1936), in other anesthetics, and after removal of the head (Child, 1910, 1929) large numbers of fissions occur, but in strychnine only 1 per cent underwent fission even after decapitation as contrasted with 54 per cent in water. Obviously, the decreased motor co-ordination and inability to crawl normally are not sufficient to explain the lack of fission, for these occur also in carbon dioxide in which fissions are increased. It seems probable that the strychnine acts in some way to suppress the development of the fission plane.

In head-frequency experiments strychnine lots show a very striking increase over controls at all levels of the body. Evidence from this line of attack indicates that strychnine action on *Euplanaria* resembles that of depressants rather than that of stimulants. No evidence was found for preliminary stimulation followed by depression such as Hinrichs (1924) obtained with caffeine. The results with strychnine resemble most nearly those obtained with chloroform and ether, which are depressants particularly of the nervous system, rather than those obtained with chloretone and KCN, which are general proto-

plasmic depressants (Child, 1916; Buchanan, 1922). That is, agents which affect the protoplasm as a whole decrease head frequency in *A* pieces by direct inhibition of the cells at the anterior cut surface and cause only slight increase in *C* and *D* pieces, whereas agents acting principally on the nervous system cause little or no decrease in *A* pieces and greater increase in *C* and *D* by blocking the inhibiting effect of the posterior section. The unusual features of the strychnine series are the fact that no decreases but occasional increases were obtained in *A* pieces and the very high head-frequency indices at all levels of the body. It is possible that strychnine blocks the inhibiting factor more completely than do other agents and therefore causes greater increases in head frequency. Block produced by strychnine has been demonstrated with the unmedullated nerves of the walking legs of the crab, *Maia squinado* (Cowan and Ing, 1935). The similarity of the results with strychnine to those with depressants agrees with Knoefel's findings (1936) that the action of strychnine on peripheral nerve fibers is purely depression.

Experiments on delay of posterior or anterior section with short pieces cut immediately posterior to the mouth also give evidence concerning this posterior inhibiting factor. Control curves agree very closely with those of Child and Watanabe (1935) on *E. dorotocephala* and Watanabe (1935*b*) on *E. tigrina*. With delay of posterior section, head frequency increases very regularly from a low value with simultaneous anterior and posterior section to 100 per cent normal heads after 24 hours' delay. This short interval before maximal head frequency is obtained indicates that, once the cells at the anterior cut surface start forming a head, the posterior cut has little or no inhibiting effect. With delay of anterior section, however, a slightly different situation exists. Head-frequency values are low after simultaneous anterior and posterior cuts, but still lower after 24 hours' delay, and then gradually increase with increasing intervals to nearly all normal by 96 hours. This decrease at 24 hours occurs after the effect of cutting has disappeared as demonstrated by oxygen and carbon dioxide determinations (Hyman, 1919*a*, 1919*b*, 1923; Robbins and Child, 1920; Watanabe and Child, 1933). Consequently, it seems probable that the activity accompanying growth and differentiation at the posterior cut also plays a part in the inhibition. In strychnine, head-frequency values are high at all times in experiments with delay of anterior or posterior section. This indicates that strychnine acts on the piece in such a way as to block the inhibiting effect of the posterior cut and also of the growth and differentiation at the posterior surface.

Another very striking point is the unusually high percentage of biaxial forms appearing in the various experimental series. This formation of heads on both cut surfaces, instead of the normal head and tail development, is another evidence of the decrease or elimination by strychnine of correlative factors within the piece. In head-frequency experiments on 1/8 pieces of 16–mm. worms, the numbers of bipolar forms are low in pieces from anterior and posterior levels and reach a peak in *E* pieces. The slight decrease observed in *C* pieces (Fig. 7) was very regular, especially in temporary exposure to high concentration in which the numbers of bipolars were highest. This may be associated with the presence of the base of the pharynx in the *C* piece (Fig. 1). Since the pharynx contains a large amount of nervous tissue, the region at its anterior end may possibly be regarded as a secondary nervous center which may to some extent reinforce antero-posterior dominance.

The high frequency of bipolar heads at the *E* level is probably correlated with the fact that this is the region of the fission zone. This region appears to be a sort of neutral zone in which dominance in either direction is slight and probably variable, that is, the

cells of this region are to some degree physiologically isolated by their intermediate position from the dominance of both anterior and second zooids. In short pieces from this region there is little differential between anterior and posterior ends; consequently, anterior head frequency is high and bipolar head frequency is higher than at other body-levels. Even in controls short pieces from this level occasionally produce bipolar heads. Because of these characteristics the region of the fission zone appears to be a particularly favorable locus for production of bipolar heads, and in strychnine, which seems further to decrease correlative factors within the piece, the highest numbers occurred in this region. Since the fission zone varies somewhat in position in different individuals, and the longitudinal gradient is slight in the second zooid, both *D* and *F* levels show relatively high bipolar frequency but not as high as the *E* level.

The curve for counts of formative cells by Curtis and Schulze (1934) on *E. tigrina* shows an interesting parallelism to that found here for percentages of bipolar forms. Although a different species, *E. tigrina* is closely related to *E. dorotocephala* and is a fissioning form. The similarity in the two curves consists merely in that peaks occur in *B* and *E* levels. However, the peak for bipolar frequency is considerably higher at *E* than at *B*, whereas those for formative cells are nearly equivalent. Work in many lines has shown that the physiological factors of distance from the dominant region, presence or absence of a fission plane, inhibiting effect of a posterior cut surface, etc., are more fundamental in regeneration than are the morphological factors which have themselves been determined in their development by previously acting correlative factors. The large amount of parenchyma tissue may be favorable for bipolar production, but physiological conditions are believed to be fundamental.

Bipolar forms have been observed by other authors in various species of *Euplanaria*, but they were commonly produced only in short pieces. Morgan (1903) found "heteromorphic heads" in short pieces from the pharyngeal region of *E. tigrina*. Child (1911*b*) described various types of biaxial forms with one or two pharynges and with or without common lateral tails. Rustia (1925) studied the effects on bipolar production of a number of agents on *E. dorotocephala* and *E. tigrina*. He also found the highest numbers at the *B* level and at the fission zone, but he used very short (1-mm.) pieces which usually give higher numbers of bipolars than do longer pieces. J. A. Miller (in press, this *Journal*) has produced bipolar forms in *E. dorotocephala* as a result of induction by transplantation. No agents other than strychnine have been reported as producing large numbers of bipolar forms in pieces as long as 1/8 of 16-mm. worms.

In experiments on delay of posterior section the number of bipolar forms in both controls and strychnine series is very high. Morgan (1903) used a delayed section experiment to test his theory that a developing head on one end of the piece influenced the other end to form a head. He found fewer bipolars, if anything, with delayed than with simultaneous cuts, but he did not have enough cases to be significant. Abeloos (1927, 1930) found that 3 days' delay of posterior section in short pieces of *Planaria gonocephala* gave approximately 1/4 bipolar forms, whereas a similar delay of anterior section produced no bipolars. In Watanabe's experiments on *E. tigrina* (1935*b*) a few bipolars appeared in 1/8 pieces from the fission zone and in delayed section experiments, but their distribution in relation to the interval of delay was irregular. In all, 52 bipolars appeared in experiments with delay of posterior section and only 17 with anterior delayed. There were also a few in the partial section experiments, especially with simultaneous anterior section and section of posterior lateral regions with delay of section of nerve cords, and

also in experiments in which one section was made under anesthesia. Since the frequencies in all cases were low, he did not attempt any conclusions with regard to their distribution. However, they seem to agree well with the experiments here reported in which larger numbers occurred.

In the controls for the strychnine series, bipolar frequency increased regularly with delay of posterior section but decreased with delay of anterior section. This increase is probably related to the fact that, when the anterior cut is made, the piece is isolated from the old head and the fission plane begins to develop. When the posterior cut is made later, this region is more active and ready to form a head. The longer the delay (up to 18 hours in these experiments) the more pieces produce heads at the posterior surface and result in bipolar forms. That the bipolar production is related to the development of the fission plane is indicated by the fact that many of the controls fissioned after the first cut was made and could not be used. Also, unpublished experiments with recently collected worms which had undergone fission and had short tails gave very few bipolar forms after delayed posterior section, suggesting that the lack of development of the fission zone was responsible. The decrease in bipolarity with delayed anterior section probably means that, during the period of development of the posterior surface under the influence of the old head, the regenerating tissue is determined as a tail and the resulting piece when later isolated is unipolar. The longer the interval of this influence, the more definite is the determination.

The occurrence of tailless forms has also been observed by Child (1912) in alcohol, KCN, low temperature, and ether. The experiments suggest that in strychnine the failure to develop tails is caused not by direct inhibition or increased scale of organization but rather by incomplete reversal of polarity. Observations of posterior regeneration in 1/8 pieces in M/100,000 strychnine sulphate (Table 2) and in M/500,000 with delay of posterior section (Table 3) reveal a continuous series from normal tails (representing no reversal) through short tails, no outgrowth, and inhibited head-forms (partial reversal) to normal heads (complete reversal). The cases of no posterior outgrowth are intermediate and represent either inhibited heads or inhibited tails, as demonstrated by the fact that some show reversal of ciliary beat at the posterior healed surface and some do not.

The experiments on rate of eye development were carried out as a means of testing whether the marked increase in head frequency in strychnine series was caused by an acceleration of development of the cells at the anterior cut surface or on the piece as a whole. Since the only difference in time was a very slight delay, it is concluded that strychnine does not accelerate head development, but decreases or eliminates the correlative factor resulting from posterior section which tends to prevent head development. The gradient in rate at different levels of the body, was in general, similar to that reported by Watanabe (1935*a*), though the differences between levels were not as great. It seems probable that this difference is related to the fact that the experiments were carried on at different temperatures. At the higher temperatures used by Watanabe (24°–28° C. as contrasted with 18°–22° C.) the rate was increased more in anterior than in posterior pieces. This was also seen in Rulon's data (1936) in which the controls of one series at 32° showed a great difference in rate between anterior and posterior pieces, whereas the other experiment at 20° showed a relatively small difference. In series treated with acids Rulon reports a considerably greater retardation, especially in anterior pieces, than that seen in strychnine. This suggests that the H-ion causes a more general protoplasmic inhibition than the strychnine, in which the effect is practically entirely on the factor inhibiting or

preventing head development. The fact that there is a small but consistent delay in strychnine indicates a certain amount of inhibitory action on the head-forming cells, but this appears to be very slight. Certainly, no stimulation was observed that could account for the great increases in head frequency and bipolarity.

SUMMARY

1. Individuals of the species *E. dorotocephala* in solutions of strychnine sulphate show greatly increased activity, spasmodic contractions, and poorly co-ordinated locomotion. They do not exhibit feeding, rheotactic, or righting reactions, and they are easily dislodged from the substratum. They show the reversal of inhibition of antagonistic muscles which is characteristic of strychnine action on annelids and arthropods but not the tetanus exhibited by vertebrates.

2. With 1/8 pieces of 16-mm. *E. dorotocephala* the head frequencies obtained by treatment with various concentrations of strychnine sulphate are consistently higher than those obtained with well-water controls.

3. In short pieces (1.5 mm.) taken from immediately posterior to the mouth, controls give very low head frequencies after simultaneous anterior and posterior section and increasing values with increasing intervals of delay of either section. Maximal indices were obtained after 24 hours' delay of posterior section and after 96 hours' delay of anterior section. In strychnine all lots gave very high values with maximum after 8 hours' delay of posterior section and 48 hours' delay of anterior section.

4. Large numbers of bipolar forms appear in 1/8 and shorter pieces in strychnine-treated lots. The largest numbers occur in the *E* pieces which are cut from behind the mouth. After 16-hour exposure to M/100,000 strychnine sulphate, 31.4 per cent of living 1/8 pieces from this level developed bipolar forms. Fewer appeared in the lower concentrations and none in water.

5. With increasing intervals of delay of posterior section numbers of bipolars formed in control groups increased from 1.1 per cent with no delay to a maximum of 26.1 per cent after 18 hours' delay. In strychnine large numbers occurred in all groups with a maximum of 73.8 per cent after 12 hours' delay of the posterior section.

6. Large numbers of tailless pieces also occurred in experimental lots. In 1/8 pieces these were most numerous in pieces cut from the anterior end of the worm. Evidence is presented that some tailless pieces, particularly from behind the mouth, were probably bipolar forms with one completely inhibited head.

7. In 1/8 pieces with continuous exposure to M/450,000, deaths were most frequent in the *A* pieces and decreased posteriorly. After short-time exposures to M/100,000, however, the deaths increased from *A* to *D* levels and then decreased.

8. In 3/8 pieces at 18°–22° C. rates of eye-pigment spot formation decreased with increasing distance of level of head development from the head of the original animal. Experimental lots exposed to M/100,000 or M/450,000 strychnine sulphate showed slightly slower eyespot formation than did well-water controls. Calculations of statistical probabilities show that the delay was more significant in anterior pieces with continuous exposure and in pharyngeal pieces with temporary exposure. No acceleration was observed.

9. In decapitated 12–14-mm. worms in M/300,000 strychnine only 1 per cent underwent fission, whereas in controls fissions were recorded in 54 per cent.

10. The data presented show that the effect of strychnine on head frequency resembles that of certain inhibiting agents such as chloroform and ether with the exception

that no decrease in head frequency occurred in *A* pieces. The observations of rates of eye development, head frequency, bipolar frequency, and effects of delayed section lead to the conclusion that the results are due to the decrease or obliteration of the effect of the posterior section rather than to the stimulation of the head-forming cells.

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A STUDY OF THE PROCESS OF FISSION IN THE NAID PRISTINA LONGISETA

(1 figure)

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MOST of the common Naididae reproduce asexually by means of fission. When the individual has attained a certain size by the addition of segments at the posterior end by the ordinary process of growth, a group of new segments is interposed in about the middle of the animal's length. These new segments are said to form a "zone of fission," and separation ultimately takes place here; the newly formed segments in front of the fission plane form the posterior end of the anterior zooid and those behind the plane differentiate into the head of the posterior zooid. The position of the fission zone is not constant for each species but may be said to be definite, within somewhat wide limits. In *Pristina longiseta* the number of segments forming the head of a zooid is constantly 7, although in Naididae generally the number is most commonly 5. The number of segments going to form the tail of the anterior zooid is indefinite, and when division is proceeding rapidly, all the intercalated new segments of the zone of fission may go to form the new head. It frequently happens that new fission planes arise in both components before separation has occurred, so that chains of individuals are produced. The whole chain of zooids is co-ordinated as regards movements, circulation, and peristaltic and antiperistaltic contractions of the intestine.

The process of fission presents a number of problems for physiological analysis. The teleological emphasis placed on the reproductive aspect of the phemonenon tends to obscure its function in the regulation of body size. The end result of the process is the production of many new individuals, but from the standpoint of the organism an interpretation based on the capacity of the individual to integrate the accretions of growth may be more objective. Growth, as manifested in the addition of new segments, and differentiation, as seen in the intercalation of new head regions, seem to offer here some causal relationship. Its experimental analysis would apparently be made easier by two circumstances: first, that the naids share with the unicellular organisms the advantage of genetically uniform individuals as a result of uniparental inheritance and, second, that the aqueous environment of the naid may be to some extent controlled. Before the crucial question of what factors determine when and where fission takes place can be answered with any degree of satisfaction, or as Adolph (1931) asks, "How does the individual organism 'know' when its proper size has been attained?" the size of an individual and the limits of its variation must be known. This apparently simple problem is of course complicated here by the larger problem of individuality, as illustrated by the physiologically integrated chain of zooids in various stages of morphological development.

Many conditions which might influence the process of fission and the possible factors responsible for observed variations in position of the zone of fission have been suggested in papers on naids. The older authors made their comments in the course of their

taxonomic descriptions, but more recently this aspect of the subject has been the exclusive concern of the paper. Bourne (1891) believed that the number of segments anterior to the fission zone was probably constant for each species. This number, which he designated *N*, would indicate the position of the fission zone, Z, which in turn will divide into two regions, Z and Z^1. Z will consist of an indefinite number of segments and form the tail of the anterior zooid, while Z^1 will consist of a definite number of segments and will become the head of the posterior zooid. "I believe that the number of segments in the sexual individual is constant for the species while that in asexual individuals is indefinite; but that *N* is a number constant for the species and that the number of segments in Z^1 is constant for the genus."

It has long been recognized, however, that *N* has no specific constancy. "In *Nais elinguis N* may vary from 12 to 20, in *Nais variabilis* from 13 to 29. Piquet furnishes numerous examples of the variability of *N* in different individuals of the same species, and not only so, but in different components of a single chain" (Stephenson, 1930).

But aside from this individual variation there is a seasonal variation. Piquet (1906) found *N* to be higher in the warm than in the cold season in each of 9 species collected by him, the average difference being 1.4 segments. Schuster (1915) came to a similar conclusion from observations on collected worms. Smith (1896) stated that *N* was larger in specimens of *P. leidyi* kept in an aquarium than in those at liberty, which presumably were in a more favorable environment. Stolte (1923), observing *N. elinguis* during the course of a year, found that *N* decreased in nature from 17 in January to 13.69 in March; it then rose rapidly and was high (17) in the sexual period (June–July), decreasing again in the latter half of the year, being between 14 and 15 from October to March. He offered no explanation for the difference between the successive January values. In *N. variabilis* there was the same rapid fall of *N* in March, e.g., from 21 to 15.8.

Stolte established laboratory cultures of *N. variabilis*, using the bacterial scum of a leaf infusion as food and from observation concluded: (1) that the position of the fission zone was dependent upon the amount of food present, *N* being small when food conditions were favorable and large when bad (unless sexuality intervened); (2) that only the rate at which division proceeded was dependent on the temperature; and (3) that both (1) and (2) were dependent on the age of the animal. In his view asexual reproduction of naids is entirely a growth process, the factor which almost alone determines the length of the zooid being the excess of nutrition present which is not needed for growth at the posterior end. This excess growth expresses itself in the formation of a fission zone, whereas age expresses itself in defective utilization of food and, like regeneration and the formation of sex organs which consume nutrition, is correlated with lengthening of the zooids.

Hempelmann (1923) has recorded with much detail an investigation of *P. longiseta* in which he made use of cultures in clones, kept in small glass dishes and fed with *Chlorella*. He believes that internal factors play a large part in determining the location of the fission zone, but he does not state what these factors are. He concludes that the position of the fission zone in *Pristina* is labile. It is determined chiefly by inner factors, but external factors may influence it within the limits imposed by the inner factors.

Eckert (1927, 1934), working with *Stylaria lacustris*, agreed with Stolte that nutrition is the most important factor in determining the position of the fission zone. The evidence for this came from a study of the location of the first fission zone in posterior zooids, in which the worms were divided into three classes: (1) those in which the posterior zooid

formed its first fission zone while still attached to the anterior zooid; (2) those in which it formed within 4 days of detachment from the anterior; and (3) those which formed it still later. It was found that the fission zone was located more anteriorly the earlier it was formed, and, by assuming that the earlier a posterior zooid formed a zone of fission the richer the amount of nutrition, it was concluded that the better the state of nutrition the more anterior is the location of the zone of fission.

Besides nutrition, age is a factor in locating the fission zone. When other factors are equal, zones tend to form in more differentiated segments and to avoid juvenile segments, by anterior displacement. This conclusion is reached from the fact that the location of a fission plane is not affected by the amount of anterior zooid cut off. From the previous conclusion one would expect a posterior displacement as a result of lack of nutrition, since the more anterior zooid removed, the less intestine would be left. Eckert concluded that nutrition and age were the only factors directly influencing the location of fission zones.

MATERIAL AND METHODS

The observations reported in this paper were made on the naid *P. longiseta*, chiefly on those grown in mass laboratory cultures. Comparisons were made, and new cultures started from time to time, with freshly collected worms from Botany Pond, a large pond on the university grounds, artificially maintained at an approximately constant level. The importance of providing a suitable supply of food, which preferably could be controlled in amount, led to a number of attempts to find a material on which the worms could feed directly. In nature *Pristina* is apparently more or less of a scavenger, living on diatoms, algae, and particularly the organic detritus of ponds. In the laboratory it thrives in protozoan cultures where, in addition to the bottom debris, it feeds upon bacteria of the surface scum. When no inert food material was found that gave adequate growth of worms, the indirect method of using protozoan cultures for food was resorted to.

Grains of wheat, previously boiled to kill the germ, provided the basic food material for the cultures. All cultures were kept in one or the other of two types of round, flat-bottomed, glass vessels with straight sides, a larger with a capacity of 7 liters and a smaller with a capacity of 3 liters. The large culture contained 3 liters of water, the smaller always 500 cc.; both were kept covered with loosely fitting glass covers. If appreciable evaporation took place, distilled water was added to bring the water level up to the mark indicating the original level. In some of the earlier experiments boiled and filtered tap water was used; in all the later work ordinary tap water was put into the open culture vessels with the wheat grains and exposed for about 48 hours. This allowed the sometimes easily perceptible chlorine to pass out of the water and furthermore got the bacterial fermentation of the wheat well under way before the culture was inoculated with Protozoa from older cultures or from the surface scum on vessels of collected waterweeds. The culture could be seeded with *Pristina* at the same time, the vessel covered, and, if the inoculations had taken, *Pristina* in quantity was available for some months.

The chief difficulty with this method of preparing cultures lay in the highly variable success of the inoculations with *Pristina*. With apparently uniform conditions the seeded worms grew profusely in some cultures and died without progeny in others. Clones were particularly hard to start for this reason.

The optimum number of grains of boiled wheat for the larger 3-liter cultures was about 20; smaller cultures seemed to require proportionally more wheat, 7–8 grains. This initial food supply was sufficient to allow a culture at room temperature to undergo its development in a period of 2–3 months and was renewed by the addition of 10 grains when it was desired to rejuvenate a declining culture. A few grains of wheat at intervals of 3–4 months was sufficient to keep cultures at low temperature alive over a period of years. Green algae were uniformly present in all old cultures.

To follow the growth and development of the *Pristina* cultures individual worms were taken with a fine-drawn pipette from the culture and inspected under the binocular dissecting microscope; an exact record was taken of the number of segments and position of fission zones, if present. The total number of worms so counted was limited in a few cases to all the worms available in the culture, but ordinarily the number of worms present was large enough to permit a random sample of 50–60 worms to suffice. After being inspected, the worms were returned to the culture. It was not found necessary to use an anesthetic to immobilize their movements under the microscope if each worm was handled individually. No attempt was made to measure the actual size of segments.

REGULATION OF SIZE BY FISSION

Dehorne (1916) divides the fission of naids into slow and rapid types. In the slow type, shown in *Dero*, only one fission zone at a time appears in the individual. After separation a period of growth follows, and when the length is sufficient, another fission zone appears. But in most naids a new fission zone appears before separation occurs. This rapid type of fission is further subdivided by Stephenson (1930) into the naidian form (*Nais*, *Chaetogaster*, *Aeolosoma*) and the Stylarian form (*Stylaria*, *Pristina*). In the former the new fission zone appears at the anterior limit of the previously produced zone, behind exactly the same number of segments from the front end of the worm as the first zone. *N*, the number of segments anterior to the fission zone, is the same in both cases, and so the third zooid, or the second individual counting from the anterior end of the worm, is composed entirely of new tissue since it contains no segment of the original worm. In the Stylarian form of rapid fission the new fission zone forms behind segment *N-1* of the original worm, the third zooid containing 1 segment—the *N*th—of the original worm, and the new fission zone is 1 segment nearer the anterior end than the first. This is repeated, shortening the body of the original worm until a lower limit of *N* is reached; then the original anterior zooid elongates by growth at its posterior end in the usual way, separation of the new zooids occurs, and, when sufficient new segments have been added, a new fission zone is intercalated in the middle and the process begins anew. According to Hempelmann (1923), the lower limit of *N* in *P. longiseta* is 12, and when this is reached, the position of the zone jumps backward 3, 2, or 1 segment.

Figure 1 represents a *Pristina* of five zooids delimited by four fission zones which were laid down in the following characteristic order. The first to appear, Z^1, arose in the fourteenth segment ($N = 14$ in this individual) and divided the worms into component zooids I and II. The second fission zone, Z^2, arose 1 segment nearer the anterior end, giving rise to zooid III. The third, Z^3, characteristically shifted to posterior zooid II, dividing it into zooids II and IV. The latest fission zone shown here, Z^4, appeared 1 segment anterior to zooid III, giving rise to zooid V. If separation were delayed and growth continued, Z^5 would be expected to arise 1 segment anterior to Z^3 and, in turn, Z^6, 1 segment anterior to Z^4, producing respectively zooids VI and VII. Addition of new segments is proceeding simultaneously with the development of new fission zones. These

embryonic segments are easily distinguished from the original segments and do not interfere with the correct counting of the old segments in such cases. The worm shown in Figure 1 can be recorded by the formula $12 - 1_3 - {}_71_9 = {}_78 - 6$, in which the dashes represent fission zones separating zooids of the given number of segments, the double dash being the oldest of the series. The figure 7 preceding zooids II and III indicates that the head segments of those zooids have attained sufficient development to show setae clearly. The subnumerals following the figures representing zooids III and V indicate the number of embryonic segments discernible. It will be noticed that only zooid I includes the 7 segments comprising the "head" in the total number of segments.

The factual data required for a study of the growth and development of a given population of *Pristina* over a period of time can be obtained only by the observation and recording of the size and condition of sufficient individuals to constitute a random sample representing the population as a whole. Heretofore, the growth and asexual reproductive activities of *Pristina* have been followed in isolated individuals and the observations recorded in the form of such formulas as that given above, or, if a comparison is made between two cultures, the data are given in the form of average number of segments per zooid, for example, with no clue as to the reliability of the statistic. In this paper all data have been condensed by calculating the following statistics for each set of observations: the mean, standard deviation, their respective probable errors, and the coefficient of variation. In addition, the number of worms making up the sample and the range of variation observed in the mean are also given.

Although these data adequately record the size of the individuals of a population, it is desirable to have a more direct measure of the amount of asexual reproduction taking place within the population. In the rapid type of fission shown by *Pristina* this is directly reflected in the number of fission zones per individual, as will be seen later. To avoid the use of too many decimals, the average number of fission zones per individual is arbitrarily multiplied by 10 to give an index of fis-

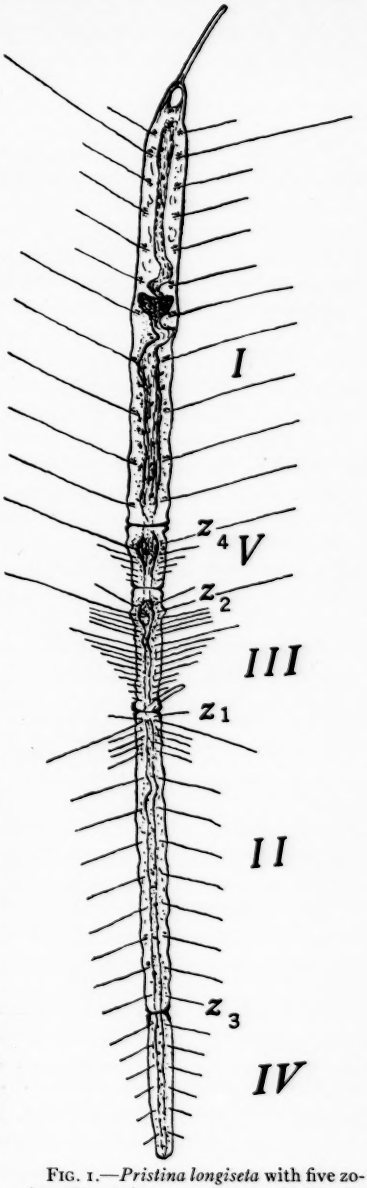

FIG. 1.—*Pristina longiseta* with five zooids. The fission zones are marked in the order of their appearance and the zooids as to their respective ages. The formula for this animal is: $12 - 1_3 - {}_71_9 = {}_78 - 6$.

sion which is useful in representing the course of events in a given population over a period of time and in comparison of different populations.

Pristina IN NATURE

If it were true that the natural environment provided a superior combination of factors which allowed the maximum expression of innate possibilities of growth and fission in *Pristina*, then a sample of worms taken from nature should furnish standards by which to judge the effects of laboratory culture itself. Such possible factors might include dietary articles or elements impossible to duplicate in the laboratory or the effect of an infinitely large quantity of culture medium as compared with that in laboratory vessels.

Some aspects of variation of size within such a sample will now be considered. These worms were collected between September 26 and October 3, 1930, when the water temperature was 19° C. The number of zooids in the various worms was distributed as follows: six zooids in 1 worm; five zooids in 22 worms; four zooids in 60 worms; three zooids in 89 worms; two zooids in 48 worms. Table 1 gives the variation in number of

TABLE 1

NUMBER OF SEGMENTS ANTERIOR TO OLDEST FISSION ZONE IN *Pristina longiseta* TAKEN FROM BOTANY POND 9/26–10/3, 1930

S.D. = standard deviation of mean; C.V. = coefficient of variation; I.F. = index of fission; No. = number of worms in sample; P.E. = probable error.

	Mean *N* and P.E.	S.D. and P.E.	No.	Range of *N*	C.V. (Percentage)	I.F.
All worms	14.16±0.04	0.88±0.03	220	12–16	6.2	22.68
2's only	13.77±0.12	1.21±0.08	48	12–16	8.8	.....
All except 2's	14.28±0.04	0.72±0.03	170	13–16	5.0	.....
Post. *N*	14.95±0.02	0.32±0.02	80	14–16	2.1	.....

segments anterior to the oldest fission zone in the 220 worms—in other words, the variation in what Bourne called *N*. The appearance of younger fission zones anterior to the oldest need not interfere with the count of the original number of segments because the oldest fission zone is morphologically the most advanced, and the embryonic segments of the newer fission zones are easily distinguished as such and ignored in the count. Granting that the introduction of younger fission zones can be recognized as extraneous, can one be reasonably certain that the *N*, i.e., the number of segments anterior to the oldest fission zone, obtained from the sample, represents the true position of the first fission zone in the original one-zooid animal? A consideration of the means by which these multizooid worms reached that condition shows that the *N* of such a sample as this is not a true *N* but must almost inevitably be too small; for, after the first fission zone appears, each new zone laid down anterior to it is 1 segment nearer the head, i.e., zone II appears in segment *N*-1, zone IV in segment *N*-2. Separation occurs at the site of fission zone I, and in both the resulting components what was zone II and III, respectively, in the original individual is now zone I in the two new individuals. To anyone attempting to determine the true position of the fission zone, the observed *N* will really be *N*-1 in this worm, and in a random sample taken from a similar population the

observed N will be a composite made up of N, N-1, N-2, etc., until a lower limit is reached beyond which fission must await the growth of new segments.

The group of 48 worms in this sample having only two zooids had an even smaller N than the sample as a whole—13.77 ± 0.12. That many of these two-zooid worms were not originally one-zooid individuals in which the fission plane had arisen free of restrictions is made probable by the fact that all the 21 worms with the fission zone in the twelfth or thirteenth segment had well-developed embryonic segments anterior to the zone, while those with the zone in more posterior segments had not. In other words, those two-zooid worms with the small N are probably separated components of rapidly dividing worms. The standard deviation of the mean N of the group, 1.21 ± 0.08, also indicates that the wide variation may be due to two different kinds of two-zooid worms having been included in the sample.

Although the observed N in this sample from nature is probably too small, there remains a possible method of determining in which segment an unrestricted fission zone will arise. Worms of four zooids or more, i.e., worms in which the third fission zone to appear has divided the original posterior zooid into two zooids, also afford excellent material on which to test the hypothesis that the position of the fission zone is determined by inherent factors transmitted by the original individual to all its daughter zooids; for the third fission zone is not subject to the negative restriction of the second, which must be placed in a segment less than the Nth, but on the contrary is apparently free, within limits, in its determination unless the daughter zooid tends to be like the parent. Of the 80 four-zooid worms of the sample there were 79 worms in which the position of the third fission zone could be directly compared with the position of the first fission zone of the same animal, and no correlation between the two could be detected. The coefficient of correlation was − 0.077 ± 0.076. This result is in strong contrast with the marked negative correlation found in similar laboratory-grown animals.

If there is no correlation between the parent and the daughter zooids, then the position of the fission zone in the latter must be determined by the same factors as operated in locating the original unrestricted fission zone in the parent zooid. In the 80 four-zooid worms of the Botany Pond sample, the mean N in the original posterior zooid, or, in other words, the segment in which the third fission zone appeared, was 14.95 ± 0.02. Its standard deviation was 0.32 ± 0.02. This probably represents the best approximation of the true N that could be obtained by observation of a sample at a given moment.

The result has been lost in the parent zooid through successive fissions and consequent separations, but in the posterior daughter zooid the result of the same factors can be seen and recorded when the third fission zone appears.

EXPERIMENTAL

A typical course of events can be described by following a laboratory culture of *Pristina* over a period of time. Table 2 condenses the results of observations on the growth and size of worms taken at intervals from such a culture. This culture, $C2_2$, was a standard 3-liter culture made up April 9, 1935, and seeded with 10–12 *Pristina* from an older culture, $C2_1$. None of these survived, so it was successfully reseeded from the same source on May 2, 1935. The culture was kept at room temperature.

Twelve days after *Pristina* was successfully introduced into the culture, the worms had increased their numbers many hundred fold. They were particularly numerous around the sides of the vessel. The figures taken from the count, made that day, indicate

the intense fission activity. The index of fission, based on the average number of fission zones per individual, is enormously high, and the number of segments anterior to the oldest fission zone averaged only 14.21. All the worms were dividing rapidly as is shown by the preponderance of multizooid types in the sample: 2 worms had seven zooids; 8 worms had six zooids; 21 worms had five zooids; 29 worms had four zooids; 7 worms had three zooids.

During this initial period of intense fission activity in laboratory cultures it was frequently observed that the normal order in which new fission zones arose was upset. Of the 8 worms having six zooids, only 1 was of the expected type $13 - 1_4 - {}_7I_{10} = {}_78 - 1 - 7$; the others showed that both the fourth and the fifth fission zones appeared anterior to the oldest fission zones, as in $13 - 1 - 1 - 1_7 = {}_78 - 6$. Similarly, four-zooid worms of the type $14 - 1 - 1_2 = {}_711$ appeared. The seven-zooid worms were as expected, $13 - 1 - 1_6{-}_7I_{10} = {}_77 - 1 - 8$ and $12 - 1 - 1_2 - 1_7 = {}_78 - 1 - 7$; these, and eight-, nine-, and ten-zooid animals are probably rare because any slight disturbance is sufficient to bring about separation at the oldest fission zone.

TABLE 2

NUMBER OF SEGMENTS ANTERIOR TO OLDEST FISSION ZONE IN *Pristina* CULTURE $C2_2$ IN SAMPLES TAKEN AT STATED INTERVALS. CULTURE STARTED 5/2/35

Date Counted	Mean N and P.E.	S.D. and P.E.	No. Worms	C.V. (Percentage)	I.F.	Range of N
5/14/35	14.21±0.12	1.51 ±0.09	67	10.6	35.37	11–16
6/6/35	15.36±0.08	0.95 ±0.06	59	6.2	4.72	12–17
6/26/35	15.80±0.06	0.663±0.05	50	4.2	3.81	14–17
8/2/35	16.95±0.13	1.42 ±0.09	58	7.5	7.73	15–20

Another abnormality observed was the presence of a normal fission zone in embryonic new segments as indicated in individuals such as $11_2 - 0_5 = {}_710 - 5$, $12 - 1 - 0_9 = {}_79 - 7$, $11_2 - 0_5 - {}_7I_{10} = {}_710 - 7$. It will be noticed that they appeared only in worms in which successive fissions had reduced N to about its lowest limit. Hempelmann (1923) states that the lowest limit of N in *P. longiseta* is 12 segments. In 11 of the 67 worms taken from $C2_2$ on the present occasion there were only 11 segments anterior to the fission zone, and in early samples from another series of cultures, in which zooid proliferation was even more intense than here, there was a total of 15 worms in which the most anterior fission plane appeared in the tenth segment. Since 7 of these segments comprise the "head" of the worm, only 3 segments of intestine were present in the zooid. These facts indicate that with very favorable environmental conditions the lowest limit of N is 10 segments. That after this limit has been reached a new fission zone develops in the immature segments at the posterior end of the parent zooid strongly suggests an escape of this embryonic region from the physiological dominance of the anterior zooid. The importance of this factor in determining the location of the fission zone will be discussed later.

In the original posterior zooid of the animal where the third fission zone, unlike the fission zones anterior to the oldest zone, is apparently free to arise in any of a number of segments, the mean N was 15.84 ± 0.06 in 49 worms. The values of N ranged between 14 and 17, and the actual amount of variation was small, the standard deviation of the

mean being 0.62 ± 0.04. When the two N's of each of the group of 49 worms are correlated, a marked negative correlation appears, the coefficient being − 0.60 ± 0.061. This result is ten times its probable error and is doubtless significant. By "Student's" method for the significance of correlation coefficients $t = -5.150$, and the probability that the result is due to random sampling is in the order of 1 to 100. In other words, in the worms of the sample under discussion, when N tends to be large in the anterior zooid, it tends to be small in the posterior, and vice versa. In a larger number of paired observations in the Botany Pond sample, no correlation obtained between the two.

In Table 2 are also shown the data of the second count of culture $C2_2$, made approximately 1 month after its start. The intense fission activity has disappeared, as shown by the worms taken at random for the sample, 59 worms with two zooids and 66 with no fission zone. In the former, the increase in N indicates that the location of the fission zone has shifted backward—a condition that has been reached either through growth of new segments at the hinder end of the anterior zooid or by the appearance of the fission zone in more posterior segments. Since only 4 of the 59 two-zooid worms gave any indication of embryonic anterior segments and only 13 had developed head setae on the head segments of the fission zone, it seems more likely that growth is proceeding very slowly and that a true N is emerging.

The third sample from $C2_2$, also shown in Table 2, continues the process of decline of the culture. To obtain 50 worms with even one fission zone, 81 worms with none were observed. The amount of variation in position of the fission zone has steadily declined as shown in the standard deviation of the mean and the variation coefficient; the limiting segments are now the fourteenth and seventeenth. Although the presence of embryonic segments at the growing posterior tip of the one-zooid animal makes an exact count impossible, a comparison can be made between the group of one-zooid worms in the sample taken June 6, 1935, and those in the present sample of June 26, 1935. The mean number of segments in the former was 24.23 ± 0.16, that in the present, 27.27 ± 0.14, which indicates that the increase in number of segments is general and not confined to N. It is interesting that there are always a few one-zooid worms with more segments than are present in some two-zooid worms. In this sample the average number of segments in both zooids of the two-zooid animals is 29.80 segments; the average number in the one-zooid worms is 27.27, but 6 one-zooid worms have 30 or more segments and 5 two-zooid worms have 28 segments.

Since this culture, if allowed to continue to decline, would not produce sufficient worms with a fission zone for an adequate sample, 20 grains of wheat were added on July 3 in order to revive the culture. This addition of food to a declining culture produces a small amount of growth and fission which never approaches that seen initially. The next and last sample was taken from $C2_2$ on August 2, following a month of hot weather; the room temperature on the day the sample was counted was 29° C. The sample consisted of 58 two-zooid and 17 one-zooid worms. The mean N of 16.95 segments was extremely high, variation was greater, but the lower value of N of 15 and the high of 20 segments indicate the great shift backward in the location of the fission zone.

This same picture of rise and fall of activity is illustrated by the course of another similar culture—$C1_2$. This was a standard 3-liter culture, made up April 9, 1935, and successfully seeded with *Pristina* on April 11, 1935. This culture will be mentioned again in connection with the effects of low temperature because the worms used to start $C1_2$ were taken from $C1_1$—a culture kept for the preceding 4 years at 10° C. Table 3

tabulates the results of observations taken at intervals on samples from $C1_2$. The culture was always kept at room temperature.

This culture was slow in getting under way; not until nearly a month after the introduction of *Pristina* were sufficient worms present to justify taking a sample. Fission,

TABLE 3

NUMBER OF SEGMENTS ANTERIOR TO OLDEST FISSION ZONE IN *Pristina* CULTURE $C1_2$ FROM SAMPLES TAKEN AT STATED INTERVALS. CULTURE STARTED 4/11/35

Date Counted	Mean N and P.E.	S.D. and P.E.	No. Worms	C.V. (Percentage)	I.F.	Range of N
5/7/35	14.42±0.10	1.20±0.07	64	8.3	25.31	11–16
5/15/35	14.58±0.08	1.10±0.06	85	7.5	17.53	12–17
6/5/35	15.18±0.06	0.62±0.04	50	4.1	4.20	14–16
6/25/35	15.61±0.06	0.60±0.04	51	3.8	3.42	14–17
8/3/35	15.41±0.08	0.89±0.06	58	5.8	6.04	13–17

once started, did not reach the heights shown in $C2_2$. Although there is no significance in the difference between the means of N in the two samples taken at a week's interval, the census of the two samples shown below confirms the indicated decrease in fissioning:

	5/7/35	5/15/35
Five-zooid worms	11	2
Four-zooid worms	16	16
Three-zooid worms	33	26
Two-zooid worms	4	41

In order to obtain a larger number of paired observations, the four- and five-zooid animals of the two samples were lumped together and the N of the anterior zooid correlated with the N of the posterior zooid of the same animal. The correlation coefficient was -0.534 ± 0.076 which, like that obtained from $C2_2$, shows decided negative correlation with a high degree of certainty. In the posterior zooid of these combined four- and five-zooid worms the mean value of N was 15.42 ± 0.06 for 43 animals. This agrees well with the 15.84 ± 0.06 value of N in the posterior zooid of similar four- and five-zooid animals in $C2_2$ but, in comparison with the Botany Pond figure of 14.95 ± 0.02, suggests that, in spite of the greater amount of fission taking place in the laboratory cultures, factors are present in nature which cause the anterior shifting of the fission zone. Temperature cannot be the factor at work, for the 19° C. of the pond water at the time the sample was taken was not appreciably different from that of cultures kept at room temperature.

The samples taken from $C1_2$ at later intervals show the expected increase in N and dropping-off of fission. The group taken June 5, 1935, consisted of 50 two-zooid animals and 69 with only one zooid; the mean number of segments in the latter was 24.35 ± 0.14. The group taken June 25, 1935, consisted of 51 two-zooid animals and 98 with one zooid;

the mean number of segments in the latter was 24.54 ± 0.17. The difference between the two groups of one-zooid worms is obviously without significance.

Since the culture was dying out, it was discontinued and the water used to start a new culture. Worms and debris of the original culture were removed by filtering through double filter paper, 20 grains of boiled wheat added, and on July 3, 1935, it was seeded with *Pristina* from $C2_2$. The results from the sample taken August 3, 1935, are shown in Table 3.

The seasonal variations in the value of N, remarked upon by many different observers, indicate the effect of temperature on the location of the fission zone in many naids. Worms collected from nature have shown N to be higher in the warm season than in the cold (Piquet, Schuster, and Stolte). It is impossible to make a direct comparison between laboratory cultures at different temperatures because the food supply is likewise affected by the temperature. Therefore, in the work reported here, advantage was taken of the very slow aging of cultures kept at a low temperature and the effects noted in worms from cultures kept for several years at low temperature. A few grains of wheat twice a year were sufficient to maintain an adequate growth of worms; all cultures showed heavy growth of green algae which may have supplemented the diet. The temperature of 10° C. was maintained by artificial refrigeration in one of a series of such low-temperature rooms in the laboratory.

Table 4 condenses the data from two *Pristina* cultures, $C2_1$ and $C1_1$, which had been kept at 10° C. for 4 years and then transferred to room temperature. Presumably because of the effect of the low temperature on the protozoan cultures constituting the initial food supply of the worms, the period of intense fission activity, which develops in the first 3 weeks of the cultures kept at room temperature, is suppressed in the low-temperature cultures. Instead of the initial rise in fissioning and the concomitant decrease in N, followed shortly by the decline in fissioning and increase in N seen in the typical room-temperature cultures summarized in Tables 2 and 3, these cultures kept at 10° C. run a more even course and age much less rapidly. The sample taken from $C2_1$ on May 28, 1934, 3 years after the culture was seeded and placed at 10° C., consisted of 52 two-zooid worms and 47 with no fission zone. The sample of $C1_1$, taken April 11, 1935, consisted of 60 worms with two zooids and 55 with one. In the worms with a fission zone, the respective N's were 14.42 and 14.68 segments. Mean values of N, representing anterior zooids as short as these, appeared only in samples from room-temperature cultures under the stress of intense fission. The absence of worms showing more than one fission zone indicates that the 10° cultures are not undergoing any such activity. Obviously, the room-temperature and 10° cultures are not of the same physiological age; the index of fission is not approximately the same. When comparisons are made between Tables 2, 3, and 4, it is even more clear that the worms kept at low temperature have shorter zooids than those kept at higher temperatures.

This conclusion is confirmed by the results of transferring the 10° cultures to room temperature. Table 4 shows the increase in N that followed the change in temperature and continued until the death of the culture. Whether any effect of the lower temperature persists as such after transfer cannot be answered. When culture 1_1, as shown in Table 4, was transferred from 10° C. to room temperature on April 11, 1935, it was filtered through double filter paper and the worms used to seed two new cultures; one of them was $C1_2$, made up in the usual way of tap water and boiled wheat, and the

other was the continuation of $C1_1$, made up of the filtered medium of $C1_1$ after it had been 4 years at 10° C. The history of $C1_2$ is given in Table 3. Table 4 continues the history of $C1_1$ which is now essentially a new culture, 20 grains of boiled wheat as usual having been added to the filtered water. Since the available worms from the old $C1_1$ were divided between the new $C1_1$ and $C1_2$, these two cultures were exactly alike, save that $C1_1$ consisted of previously used water.

TABLE 4

NUMBER OF SEGMENTS ANTERIOR TO OLDEST FISSION ZONE IN 2 *Pristina* CULTURES KEPT FIRST AT 10° C., THEN TRANSFERRED TO ROOM TEMPERATURE

DATE COUNTED	MEAN N AND P.E.	S.D. AND P.E.	C.V.	I.F.	LIMITS OF N	NO. OF WORMS
Culture 2_1 started 4/20/31; put at 10° C. 5/2/31						
5/28/34	14.42±0.07	0.77±0.05	5.3	5.25	12–15	52
Transferred to room temperature 9/11/34						
10/17/34	15.41±0.07	0.74±0.05	4.8	3.83	13–16	46
4/9/35	16.09±0.16	1.73±0.11	10.7	5.88	14–22	53
6/4/35	16.38±0.08	0.77±0.05	4.7	5.11	15–18	45
Culture 1_1 started 4/25/31; put at 10° C. 4/23/31						
11/17/31	15.01±0.07	0.91±0.05	6.0	10.0	13–16	72
4/11/35	14.68±0.10	1.13±0.07	7.5	5.21	11–17	60
Transferred, after filtering, to room temperature 4/11/35						
5/1/35	15.11±0.06	0.66±0.04	4.3	7.94	14–16	54
6/3/35	16.78±0.05	0.50±0.03	3.0	5.93	15–18	51
6/24/35	16.71±0.07	0.80±0.05	4.8	7.53	15–18	55
8/6/35	17.53±0.23	1.38±0.16	8.4	2.14	15–20	18

Table 3 shows that culture $C1_2$, seeded with worms from a stock kept for the 4 preceding years at 10° C., immediately began the intense fissioning introductory to the normal cycle of culture activity. Table 4 shows that culture $C1_1$ failed to respond to the stimulus of a fresh supply of food by the normal rapid proliferation of zooids. The sample taken May 1, 1935, 2 weeks after the culture was well under way, consisted of 54 worms of two zooids and 14 with one. One month later, N was very large and the number of worms on the decline. Ten grains of wheat added June 4, 1935, produced some rejuvenescence, but on August 6, 1935, the few worms remaining gave the largest mean

N recorded in this work. Such results are taken to indicate the accumulation of substances in the water of long-continued cultures which are deleterious to the growth of *Pristina* and so lead to the suppression of fission.

In the rhabdocoel turbellarian *Stenostomum leucops*, it was found that an anterior cut through the head region which removed the cephalic ganglia from the body of the worm produced the appearance of a fission zone in 80 per cent of the worms so treated as compared with 10 per cent in control worms (Van Cleave, 1929). A similar treatment of *P. longiseta* was attempted. One-zooid worms with sufficient segments to be expected to produce a fission zone shortly (26–30 segments) but with no morphological indication of its appearance as yet were selected and paired. Five of the 7 head segments of the one member of the pair were removed and both worms isolated in filtered water. The headless worms regenerated the missing head segments in 4–5 days, but in no case did a fission zone arise during this period. Of the normal controls a fission zone made its appearance within 48 hours in 50 per cent of the worms. In similar pairs of two-zooid worms with an early fission zone already present removal of 5 head segments resulted in resorption of the early fission zone and regeneration of the missing segments.

The unoperated controls either separated as the fission zone matured or remained attached during the period of observation; in no case did a second fission zone develop. *Pristina* is apparently too highly differentiated in its organization to offer any comparison with the labile fission zone of *S. leucops*.

DISCUSSION

The process of fission in *Pristina* is clearly indicated to be the result of physiological isolation (Child, 1924, 1928, 1929). Just as physical isolation of small parts of the simpler animals is followed by the reconstitution of a complete new individual, so the escape of a region from the dominance of the head and nervous system may lead to the differentiation and development of a new individual before actual physical separation occurs. The condition of dominance established by the anterior end of the zooid over its subordinate posterior parts is directly related to a physiological gradient of metabolic activity, the high end of which is the dominant region of that gradient. The cerebral ganglia and ventral nerve cords with their segmental ganglia are one morphological expression of this gradient and exercise to some degree a functional control over posterior zooids. The head of each new zooid formed is the high end of an integrative gradient representing that individual zooid (Hyman, 1916). The range of dominance, as shown by the distance of the fission zone from the head in each new fission, is closely correlated with the state of development of the head and nervous system in that zooid. In the typical five-zooid worm shown in Figure 1 the zooid in which development of the head is most advanced has attained the greatest length before a fission zone appears, and the earlier the stage of development of the head the shorter the zooid at the time when fission begins.

Physiological isolation and, therefore, the appearance of a fission zone normally result from increase in length in *Pristina*. This increase through the addition of new segments at the posterior preanal segment of the zooid brings a certain region beyond the range of dominance of that individual. The data indicate that under some conditions, notably low temperature, physiological isolation has been brought about by a decrease in dominance.

According to this interpretation of fission in *Pristina*, such factors as nutrition, tem-

perature, and age of culture influence the position of the fission zone only because they alter in some way the range of dominance of the head and nervous system. The operation of these factors can be summarized from the data as follows.

1. With good nutrition at room temperature a population of *Pristina* is characterized by short anterior zooids, very rapid fission, long chains of zooids, and large segments. All these characteristics are indicative of rapid growth and would be expected to follow as a result of the rapid addition of large amounts of embryonic tissues. It has been seen above that in a population composed exclusively of multizooid animals there is good reason to believe that the apparent anterior shift in the position of the oldest fission zone of the chain is the result of rapid fission and subsequent separation and does not represent the position of an unrestricted fission zone in an originally one-zooid individual. Such a condition obtains, however, in the original posterior zooid found in four- or five-zooid animals, and there the fission zone has shifted posteriorly. The reason for the slower development of the original posterior zooid in such rapidly proliferating worms is undoubtedly related to the smaller amount of nutrition available to it, as compared with the anterior zooid. All the food enters the gut through the mouth of the anterior zooid, and to judge from the larger size of the segments of the zooids anterior to the oldest fission zone, most of it is absorbed there. Consequently, the growth of the posterior zooid is relatively retarded, and the dominance of its head keeps pace with the differentiation of new segments and so extends over a greater distance. Anterior to the oldest fission zone, the relation of dominance to the rapidly added new segments is quite different. The intense activity accompanying the rapid proliferation of embryonic segments at the posterior end of each new zooid and the decrement in transmission of dominance because of the low conductivity of the embryonic tissues for excitation combine to isolate segments closer and closer to the head. Some of the results of almost complete lack of dominance under conditions of intense growth activity were described above. Two or even three fission zones arose successively in the anterior zooid instead of alternating between anterior and posterior zooids as appears normal with a slower growth rate. A fission zone appeared in the midst of a group of embryonic segments apparently as a result of their escape from the dominance of the head of their zooid.

The combination of ample food, favorable temperature, and fresh culture medium produces rapid growth of new segments. This increase in length and local activity leads to physiological isolation of regions which thereupon differentiate independently to form new zooids. There is no evidence here of any decrease in dominance.

2. With poor nutrition at room temperature, cultures of *Pristina* are characterized by long zooids, slow fission, short chains of zooids or one-zooid animals, and small segments. There is little indication of growth here. New segments are added at a rate that permits complete dominance of the zooid by the head end until a limit to the extension of dominance is reached through gradual increase in length. As the culture grows older this limit, shown by the position of the fission zone, becomes greater.

3. With good nutrition at a temperature of 10° C. a population of *Pristina* is characterized by short zooids, slow fission, short chains of zooids, and small segments. The approximate 10° decrease in temperature has apparently brought about a decrease in growth rate, similar to that seen in cultures with poor food conditions at room temperature. The striking difference between the two is found in the position of the fission zone. At 10° C. the anterior zooid is very short and not subject to the objections raised against the observed short anterior zooids of multizooid worms. The short anterior

zooid found in low-temperature cultures is also characteristic of naids taken from nature in the cold season. It is suggested that the low temperature has adversely affected the activity of the dominant anterior end of the zooid so that physiological isolation results from a decrease in dominance. When a low-temperature culture showing the typical short anterior zooid is transferred to room temperature, the anterior zooid becomes longer; in other words, the new fission zones begin to arise more and more posteriorly, presumably as a result of an increase in range of dominance of the head end.

4. Certain data indicate that, when a culture of *Pristina* is maintained in the same medium over a period of many months, there is formed or accumulates in the culture medium a substance or substances inhibitory to the growth of the worms. New cultures made with "used" or "conditioned" water and kept at room temperature gave indication of ample food, and yet fission and growth were very slow as compared with the activity in control cultures. Very long anterior zooids characterized the last stages of cultures with "conditioned" water kept at room temperature. The same inhibitory factor may be responsible for the failure of old room-temperature cultures to undergo more than slight rejuvenescence on the addition of more food.

SUMMARY

1. The position of the fission zone in *P. longiseta* can be altered by such environmental factors as state of nutrition, temperature, and age of medium.

2. Conditions favorable to the rapid growth of new segments—such as ample food, temperature of 20–25° C., and fresh culture medium—produce intense fissioning activity in the course of which the fission zones shift anteriorly.

3. With poor nutrition at the same temperature the fission zone shifts posteriorly, giving long zooids.

4. With good nutrition at a temperature of 10° C. the fission zone shifts anteriorly, giving very short zooids.

5. Worms grown in previously "used" or conditioned water at 20–25° C. and ample food had very long zooids.

6. The process of fission is regarded as an instance of physiological isolation and, consequently, the environmental factors as influencing its position by altering the range of dominance of the head end of the zooid.

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AXIAL DIFFERENCES IN WATER-ABSORBING PROPERTIES OF OLIGOCHAETE TISSUES

(Two figures)

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POLARITY in the oligochaetes is indicated by orderly antero-posterior differences in many physiological and morphological features. With some exceptions, e.g., *Aelosoma hemprichii* (Hyman, 1916) it has been found that the anterior and posterior regions are relatively more active than the mid-region. This type of physiological pattern has been called a double gradient, in distinction to the single gradient exhibited by such forms as *A. hemprichii* and the young zooids of the Naididae, in which activity decreases antero-posteriorly. Evidence for the double type of gradients, based upon quantitative and physiological analyses, may be summarized as follows: susceptibility to toxic agents (Hyman, 1916), regeneration potential (Hyman, 1916), orientation in an electric current (Moore and Kellog, 1916; Hyman and Bellamy, 1922), oxygen intake and carbon dioxide output (Hyman and Galligher, 1921; Shearer, 1924), sensitivity to light and in the distribution of photoreceptors (Hess, 1924, 1925; Nomura, 1925), distribution of pigment (Pickford, 1930), distribution of extractable sulphydryl (Perkins, 1929), distribution of oxidizable substances and carbon dioxide production (Watanabe, 1931), H-ion concentration and colloidal dispersion (Růžička, 1927), water content and heat rigor (Hatai, 1924).

Attempts to disclose the fundamental nature of polarity by the application of external agents encounter two problems: the action of the agent on the living protoplasm, and the way in which the protoplasmic system varies along the axis. Much insight into the former is necessary before interpretations of the latter are valid. The present work represents an attempt at analysis by a study of the action of certain agents that are known to affect the water content of tissues.

THE PROBLEM

Since water forms the largest constituent of protoplasm and has an important role in the physiological activity of living organisms, animals which show an axiate differential in respiratory rate, in susceptibility, and in the rate of regeneration may be expected to exhibit a similar differential in the distribution of water. The present investigation was undertaken for the purpose of determining whether, in annelids, tissues histologically approximately identical, but located at several regions along the antero-posterior axis, manifest an orderly differential in water content, and whether such differences express a gradient in the physiological sense employed by Child. The major problem leads to a second question, namely, do the tissues along the axis differ with respect to their ability

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to appropriate water? Evidence was therefore sought as to whether axial differences in the effect of lipoid solvents, of the Ca-ion, and of calcium precipitants on water appropriation exist in tissues of the same type. It was considered possible that such evidence might indicate something of the mechanism of water uptake in general, and of the quantitative differences of this mechanism along the axis.

Four specific objectives were set: (1) to ascertain whether in the oligochaetes *Lumbricus terrestris* and *Helodrilus* (= *Allolobophora*) *caliginosus* a differential in water content exists in tissues taken from different regions of the long axis, and whether the conditions in these two American species, in which growth takes place by the addition of metameres anterior to the anal segment, are in any way comparable to conditions in the two Japanese species *Perichaeta divergens* and *P. megascolidiodes* (Hatai, 1924). The latter two species grow by a passive elongation; the number of metameres does not increase with the growth in length of the body but is fixed early in development; (2) to determine the responses of tissue from the several regions to hypotonic environment; (3) to test whether identical tissues from several regions differ with regard to their susceptibility to lipoid solvents, calcium precipitants, and the Ca-ion itself; and (4) to determine possible differences between old and young animals.

MATERIALS AND METHODS

The specimens of *H. caliginosus* were collected on the Northwestern University campus and Cook County Forest Preserve in the fall and early spring of 1930–31 and 1931–32. They were used immediately or very soon after they were brought into the laboratory. The specimens of *L. terrestris* were collected mainly in Wilmette during the fall and early spring of 1932, 1933, and 1934. The animals collected in the fall were kept during the winter in a large box filled with rich humus. Occasionally it was necessary to secure material from a dealer, but these worms responded to analysis in every respect similarly to material collected locally.

The nature of the problem made it necessary to formulate five groups of experiments. The first group (I) was planned to investigate the distribution of water in the anterior, middle, and posterior regions of the earthworms; the second group (II) to ascertain the effect of distilled water on tissues from similar levels; the third group (III) for the purpose of determining the nature of the effect of the Ca-ion on the water appropriation by the tissues; the fourth group (IV) for determining the action of potassium oxalate solution; the fifth (V) to study the effect of a lipoid solvent on water appropriation in histologically identical tissues from the three regions. A total of 550 animals was employed for these five groups—50 adults of *H. caliginosus*, 100 of *L. terrestris;* 50 adult and 50 young animals were used in the first group, 50 adults of each species in the four remaining groups.

Group I.—The procedure for Group I was as follows: Portions, about 15 segments in extent, were cut from three regions—from the extreme anterior end exclusive of the prostomium, from the region immediately posterior to the clitellum, and from the extreme posterior end. The prostomium and the clitellum, since they are highly specialized structures, were discarded in all cases. The digestive tract, nerve cord, and other internal organs were removed. Small portions of the body wall, composed largely of muscle tissue, were thus secured, and all adherent water and body fluids removed by pressing them between blotting paper. The tissues were then placed on small watch glasses and weighed to one-tenth of a milligram. The glasses containing the tissue were

then placed in a desiccator over sulphuric acid (C.P.), until the dry tissues had attained constant weights, and the amount of "free" water, i.e., water removable by sulphuric acid, in the tissue was obtained. Because chemically pure sulphuric acid has constant water-absorbing properties at constant temperature and pressure, errors arising from differences due to variations in temperature in sand and water baths were avoided. The procedure was followed for the anterior, middle, and posterior portions of each animal singly.

TABLE 1

SUMMARY OF DATA

Average of 50 animals for each experiment. Desiccation over sulphuric acid at room temperature (in percentage)

	Anterior	Middle	Posterior
Water content:			
Helodrilus caliginosus	78.1 ±0.172	79.1 ±0.11	80.1 ±0.1001
Lumbricus terrestris	79.8 ±0.0078	81.7 ±0.065	82.24±0.042
L. terrestris (young animals)	78.6 ±0.24	78.76±0.165	78.5 ±0.349
Appropriation of distilled water:			
H. caliginosus	66.7 ±0.62	30.2 ±0.095	46 ±0.639
L. terrestris	40.43±0.3005	22.6 ±1.68	16.1 ±0.0515
Appropriation in M/1,000 calcium chloride solution:			
H. caliginosus	29 ±0.19	2.6 ±0.143	1.06±0.173
L. terrestris	24.1 ±0.358	0.33±0.776	1.85±0.264
Appropriation of M/1,000 potassium oxalate solution:			
H. caliginosus	39.7	46.1	64
L. terrestris	60.02±0.73	66.05±0.98	69.1 ±0.806
Appropriation of ethyl alcohol solution:			
M/1,000 H. caliginosus	56.7 ±4.39	52 ±0.65	59.3 ±0.0328
0.5 per cent L. terrestris	62.5 ±0.93	25.6 ±0.3	44.2 ±0.829
1 per cent L. terrestris	46.6 ±0.99	31.2 ±1.038	31.8 ±1.001

The procedure in handling the young specimens of *L. terrestris* (3–5 cm. long) was identical with the foregoing, except that each animal was divided into three equal parts, the prostomium only being discarded.

Group II.—The animals, exclusive of the prostomium and clitellum, were divided into three approximately equal portions. As in Group I the body wall alone was used, but in this and in succeeding experiments the wall of the entire region was included. To avoid errors due to differences in hydrion concentration, all distilled water used for this group of experiments was brought to pH 6.8–pH 7.0 by aerating with CO_2 free air. The tissues from the several regions were prepared as described above, weighed, and immersed in distilled water. The original H-ion concentration was maintained during exposure by frequently changing the water. It was found that 6 hours was required for the tissue to attain maximum weight. The tissue was then removed, pressed between blotting paper, weighed, and placed in a desiccator until a constant weight was attained. This required

from 2 to 3 weeks and involved weighings at intervals of several days. The difference between the weight immediately after the removal from distilled water and the final weight was considered as due to changes in the water content, as the result of an osmotic unbalance between the tissues and distilled water.

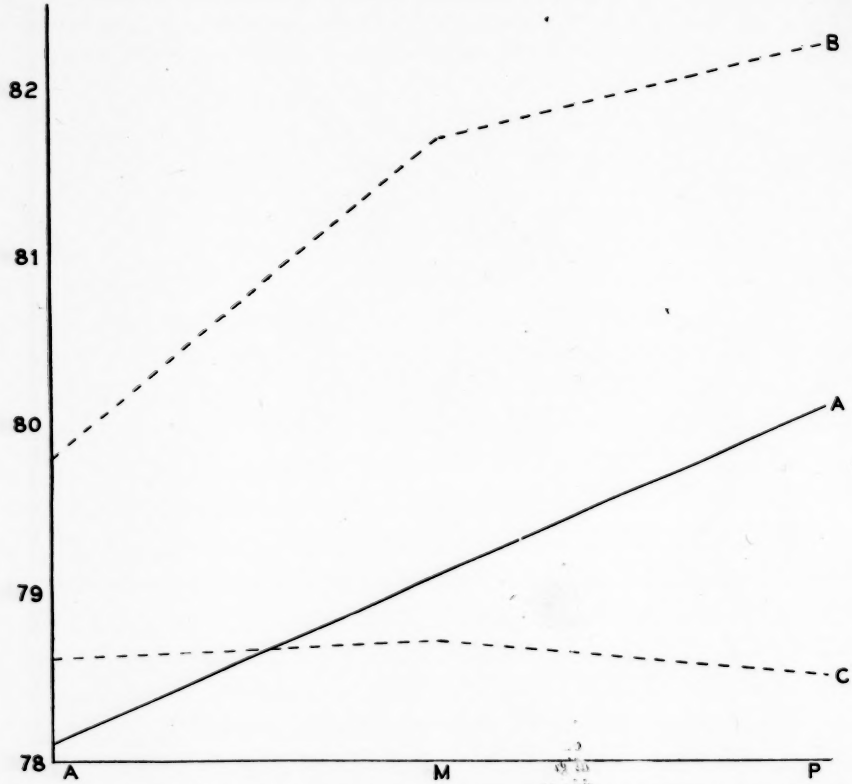

FIG. 1.—Amount of water in percentage of total body weight (ordinate) in the anterior, middle, and posterior regions of the animals (abscissas). Desiccation over sulphuric acid (C.P.). Average of 50 animals for each experiment: *A*, *Helodrilus* (=*Allolobophora*) *caliginosus*, ——; *B*, *Lumbricus terrestris*, - - - -; *C*, *L. terrestris*, young animals, - · - · -.

Group III.—A slight trace of calcium in a solution has frequently been found to decrease the permeability of the membrane to substances entering and leaving the cell (see Beutner, 1933, p. 82, for a summary)—an action said to be due to the dehydrating effect of the Ca-ion on the tissue proteins. Any differential in the imbibition of water in a calcium solution would be an indication of a differential in calcium metabolism along the axis. A solution of M/1,000 calcium chloride was employed in this group of experiments. This concentration had no appreciable effect on the hydrion concentration of the solution.

Group IV.—This group of experiments was designed to test the possibility that differences in the concentration of tissue calcium play a role in determining differences in the water content along the axis of an animal. Potassium oxalate is a precipitant of calcium; therefore, any differential in the imbibition of the solution by the tissues of the different regions after exposure to the oxalate is indirectly indicative of a difference in the calcium content along the axis. A solution of M/1,000 potassium oxalate was used for this set of experiments, the procedure being the same as in Group II. This small addition

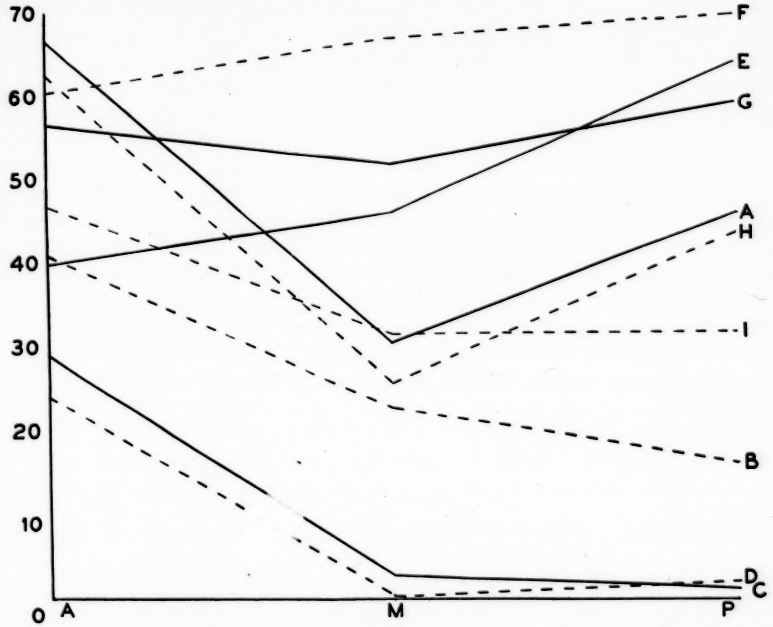

FIG. 2.—Percentage of water appropriated (ordinate) by the anterior, middle, and posterior regions of the animals (abscissas). Desiccation over sulphuric acid at room temperature. Average of 50 animals for each experiment. *Helodrilus caliginosus*, ——; *Lumbricus terrestris*, - - - -; *A* and *B*, distilled water; *C* and *D*, M/1,000 calcium chloride solution; *E* and *F*, M/1,000 potassium oxalate solution; *G*, ethyl alcohol, M/1,000; *H*, 0.5 per cent ethyl alcohol solution; *I*, 1 per cent ethyl alcohol solution.

of the oxalate slightly decreases the hydrion concentration of the solution, the decrease being approximately pH 0.2. The osmotic pressure of such a low concentration is only slightly above that of distilled water.

Group V.—Ethyl alcohol was employed in this group of experiments because of its lipoid solvent properties. According to Heilbrunn (1928), Buchanan (1935), and others, tissues absorb water in the presence of lipoid solvents, presumably because of the leaching-out or the dissolving of cell lipoids. Differences in the effect of a lipoid solvent on water uptake along the axis are to be considered suggestive of a differential in lipoid distribution in the tissues. A solution of 0.5 per cent ethyl alcohol was employed on 25 *L. terrestris*, and on another group of 25, 1 per cent alcohol was used.

DATA

The data obtained were reduced to percentage of total fresh weight per gram of tissue either lost or gained during the course of an experiment. In each group the probable error was calculated, and the statements hereinafter made are based upon the significance of the data treated in this manner. In this report the data have been converted into graphs. The original data are on file in the library of Northwestern University.

The percentage of water in the anterior, middle, and posterior regions of the animals shows a distinct antero-posterior gradient; the posterior end has the highest percentage of water, the middle less, and the anterior end least (Table 1; Fig. 1). In young animals the water content does not vary appreciably along the axis.

Upon exposure to distilled water for 6 hours anterior tissues appropriate more water (gain more weight) than do similar tissues from middle and posterior regions (Table 1; Fig. 2). Since the tissues are histologically indistinguishable, differences in water appropriation must be attributed to osmotic relations of the tissue.

M/1,000 calcium chloride solution decreases water uptake, particularly in the middle and posterior regions of the animals. In no case did the increase in weight exceed more than 3 per cent of the total fresh weight, and in certain isolated cases these tissues lost weight. Moreover, anterior tissues did not gain so much weight as did anterior tissues in distilled water.

The appropriation of water in M/1,000 potassium oxalate is in the reverse order to that in M/1,000 calcium chloride. This suggests a differential in the distribution of calcium in the tissue, since potassium oxalate inactivates calcium. Apparently middle and posterior regions contain more active calcium than does the anterior region.

The order of water appropriation in ethyl alcohol solution is the same whether the solution is on the order of 0.5 or 1 per cent; the anterior region in all cases imbibed the most water. Since both distilled water and lipoid solvents are said to dissolve or leach out lipoids, the results may be related to those obtained by the use of distilled water alone.

DISCUSSION

Histological preparations show that various regions of the body wall employed in these experiments are, so far as can be determined, histologically quite the same. The data demonstrate that such histologically similar tissue from the earthworms *H.* = *A. caliginosus* and *L. terrestris*, derived from different regions of the animals, lose different proportions of total weight when brought into a chamber containing sulphuric acid. Drying over sulphuric acid does not remove all the water from the system, but the proportions remaining must be very small, since the tissue attains a constant weight within 2–3 weeks, and the residue is hard and brittle. Thus in tissues from the various regions of these annelids the quantities of extractable water are different.

Various methods have been devised to determine the percentage of free and bound water in inanimate colloidal systems and in living tissues. From the results of the techniques which have been used it is evident that any definition of free water is determined by the nature of the particular experimental procedure upon which the definition is based, for the methods devised do not measure the same property. Gortner (1930) states:

To be sure, there is no good definition of bound water, no exact physico-chemical technique which enables us to differentiate sharply the properties of free water from those of bound water, and it seems highly probable that there is an insensible gradation from water which is an integral part of the protoplasmic structure to that which is simply the medium in which protoplasm is suspended.

These data show only the portion that may be drawn off by sulphuric acid. The data (Fig. 1) show that in the tissues of the earthworm the proportions of abstractable water and dry substance differ in the different regions.

The water content along the long axis of *L. terrestris* and *H. caliginosus* as shown by these data is different from that given by Hatai (1924) for the water content in the Japanese species *P. megascolidiodes* and *P. divergens*. In the Japanese species the middle pieces showed the highest percentage of water, the posterior pieces less, and the anterior pieces least. The divergent findings may be due to different physiological characteristics of the species employed, more especially by differences in the manner of growth. The American species, employed in the present investigation, have a region of high growth activity at the posterior end, for it is here that new segments are added. This tissue is undifferentiated and physiologically young as compared with more anterior regions (Stephenson, 1930). In the Japanese species growth takes place by an elongation of segments already present, for the number of segments is fixed early in development. It is also possible that the apparent conflict of results may be due to the different methods employed. Hatai placed the body-wall tissue in a hot-water oven (temperature, 95°–100° C.) for a week. Easily volatile substances, as well as water in its several states, may be removed from the tissue by heating in a hot-water oven. The method used in this investigation was drying in a desiccator over sulphuric acid for 2–3 weeks. The water-absorbing power of sulphuric acid (C.P.) is constant at constant temperature and pressure, withdrawing only the water from the system in proportion to a partition coefficient between the acid and the tissue.

Maloeuf (1935) reports that in the earthworm there is a rise in solids and total organic material in terms of the living weight from the anterior end to the clitellum, with a continuous decline posteriorly. A critical statement concerning his findings is impossible since the published data are in abstract form, and no mention is made of the technique employed.

Live tissues from different regions appropriate water when placed in distilled water. If the living cell is placed in distilled water, it expands or at least tends to expand (Heilbrunn, 1928; Kedrowsky, 1931). Lillie (1916) found that fertilized *Arbacia* eggs in hypotonic sea water imbibed more solution than did unfertilized eggs. Northrup and Kunitz (1927) calculated the value of this osmotic pressure and were thus able to derive the molecular weight of the proteins involved. The entrance of water into the egg follows the same course as the entrance of water through a collodion membrane. Buchanan (1930*a;* 1930*b*) reported that the exposure of *Euplanaria* to distilled water is accompanied by an increase in weight. From many lines of evidence (Hofmeister, 1891; Overton, 1895; Loeb, 1922; Mudd, 1925; Sorensen, 1924; Northrup and Kunitz, 1927; Beutner, 1933) it may be concluded that imbibition in distilled water is in part conditioned by the osmotic pressure between the solution and the contents of the cell; by the ionic equilibrium of the solution, the membrane, and the interior protoplasm; and by the physical and chemical composition of the membrane.

It has been frequently shown by a number of investigators that the disintegration of cells and tissues is accompanied by distension and swelling (Lillie, 1916; Buchanan, 1929). Buchanan found that in KNC solution the visible differential of disintegration of *Amblystoma* embryos is in some way related to the water content—the higher the water content, the greater the susceptibility. Highly susceptible regions imbibe more water than do less susceptible regions. The results on the differential imbibition of distilled water by the tissues suggest that differential susceptibility, as shown by disintegration, may be an expression of a differential in water content along the axis, or at least of water acceptance.

The H-ion concentration of a solution is a condition affecting tissue susceptibility, as evidenced by swelling and disintegration. Růžička (1927) has shown that a differential in hydrion exists along the axis of the earthworm. This fact suggests that the H-ion concentration of the tissue may play a role in controlling the water content along the axis. The results here show that regions which have the greatest dry weight imbibe the greatest amount of distilled water; according to Růžička these regions also have the greatest tissue acidity.

Distilled water has been shown to dissolve, redisperse, or leach out lipoids. Hansteen-Cranner (1919, 1922) states that distilled water can cause leaching of the protoplasmic lipoids of various plant tissues. Runström (1923) has presented evidence to show that lipoids in the sea-urchin egg are dissolved when the egg is placed in hypotonic solution. Heilbrunn (1928) found that, when the sea-urchin eggs are exposed to solutions containing a high percentage of distilled water, the fat particles, which showed plainly when the eggs were centrifuged, are dissolved. These facts suggest that the differential in the uptake of water by earthworm tissues from the several regions in distilled water may have been the result, at least in part, of a differential distribution of lipoids in the tissue.

From the data one may conclude that the presence of an excess of the Ca-ion disturbs the process of water uptake (Fig. 2). In the calcium chloride solution there was a marked decrease in water appropriation as compared to distilled water. Further, the action of calcium on the tissues from the various levels of the earthworm results in differences in their water uptake; specifically, the anterior region appropriates more than the middle or the posterior tissues. One may assume that the Ca-ion acts in the same manner on each level; therefore, differences in water uptake must be attributed to axial differences in one or more of the physiological processes in which calcium plays a role.

Potassium oxalate accelerates the uptake of water, very probably because of its specific effect as a calcium precipitant. In *Euplanaria*, also, swelling followed by cytolysis occurs in potassium oxalate solutions (Buchanan, 1935).

The results here suggest the possibility that the proportion of active calcium varies along the axis, for the tissues from the different regions do not behave in the same manner when exposed to the same concentration of potassium oxalate. Tissues from the posterior-end imbibed the greatest, and tissues from the anterior end imbibed the least, water. These facts indicate that active calcium may be present in larger amounts in posterior than in anterior tissues.

The results obtained by employing ethyl alcohol solution may be attributed to its action in dissolving tissue lipoids. The data (Fig. 2) suggest that in these annelids the distribution of lipoids varies with the region, being highest at the anterior end, less at the posterior end, and least in the mid-region. The data on water uptake in alcohol solutions given here suggest a possible origin of the results obtained when the tissues were exposed

to distilled water, since one of the possible effects of distilled water is the leaching-out or redispersion of lipoids (Bayliss, 1927).

Results in the experiments of Groups II, III, IV, and V cannot be ascribed to differences in the H-ion concentrations of the solutions employed; the distilled water employed was nearly neutral, pH 6.8–7.0; M/1,000 potassium oxalate solution increased the acidity only slightly. The hydrion concentration of M/1,000 calcium cholride solution and 0.5 and 1 per cent ethyl alcohol solution was not appreciably different from that of distilled water. The osmotic pressures of the solutions used were only slightly above that of distilled water and may be considered hypotonic with respect to annelid tissues. Within any single experiment the osmotic pressure and the H- ion concentration were constant for pieces from all three regions. It seems clear that the differences in the behavior of the tissues with respect to water gain or loss are the consequences of their difference in osmotic activity, calcium concentration, and the distribution of lipoids.

In general, the data obtained on the water relations of the two species of annelids are in agreement. The total water content of *H. caliginosus* is slightly lower than that of *L. terrestris*. In the latter, the difference in water content between the middle and the posterior regions is less than the difference between the same regions in *H. caliginosus*. The distribution of water in both species expresses a gradient with an increasing amount of water from the anterior to the posterior end. Van Deursen, in unpublished work done in this laboratory, employing similar technique in which 106 animals were studied, has secured data of the same nature.[2] In young animals the distribution of water does not express a gradient as in mature forms, for the water content is the same in all regions. The fact that the results on water content in Group I, where only the fragments of tissue from the three regions were used, are similar to those of Groups II, III, IV, and V, where the entire region was used, indicates that the differences are due to the condition of the tissue rather than to the surface exposed. The use of both small and large pieces also eliminates the possibility that differences might have been due to the differences in traces of blood retained in the tissues.

As stated above, the amount of water appropriated by the tissues, when exposed to the various solutions, is dependent not only on the type of solution employed but also on the region from which the tissue is taken. The amount of distilled water imbibed in both species of oligochaetes is greatest in pieces from the anterior end. The posterior region of *H. caliginosus* gains more than the middle region; the middle region of *L. terrestris* gains more than the posterior region. This indicates that the two species differ distinctly with regard to the important physiological processes in which water is involved. In the experiments in which M/1,000 calcium chloride solution was used, the greatest percentage of solution was imbibed by the anterior tissues in both species, the least by the posterior tissues. In fact, the posterior segments of *H. caliginosus* lost weight. In potassium oxalate solution the posterior pieces of *H. caliginosus* and *L. terrestris* gain the most weight, the middle pieces less, and the anterior pieces least. The concentration of ethyl alcohol solution affects the amount of uptake by the tissue but has no effect on the order of appropriation by the tissues from the various levels. The anterior end gains the most weight, the posterior end less, and the mid-region least. At present no explanation can be offered for the fact that the amount of appropriation by the tissues of *L. terrestris* is greater with a 0.5 per cent concentration than with a 1 per cent concentration of ethyl al-

[2] Master's thesis of Arthur Van Deursen, "Mineral Ash in the Body Wall of Annelida," on file at the Deering Library, Northwestern University.

cohol, or the fact that appropriation by the tissues of *H. caliginosus* is less with a M/1,000 ethyl alcohol solution than with distilled water alone. The results show that the tissues of these earthworms appropriate water when placed in distilled water; the Ca-ion decreases and potassium oxalate increases the uptake of water; ethyl alcohol, a lipoid solvent, favors water uptake. The amount of appropriation varies with the region from which the tissue is taken.

The differential in water content and water appropriation in solutions of calcium chloride and potassium oxalate in the three regions of the earthworms used expresses a primary type of gradient. The gradient in water appropriation in distilled water and in ethyl alcohol solution is of the secondary type. This fact suggests that gradients of the secondary type, which have been previously described for annelids (Morgan and Dimon, 1904; Hyman, 1916; Moore and Kellog, 1916; Hyman and Galligher, 1921; Hyman and Bellamy, 1922; Hatai, 1924; Hess, 1924, 1925; Růžička, 1927; Watanabe, 1931), may in some way reflect axial differences in lipoids, while primary gradients are more closely related to calcium distribution.

The results of this investigation show that fundamental differences, not associated with observable histological differences in the various regions—such as water content, reaction to hypotonic environment, lipoid solvents, calcium precipitants, and the Ca-ion itself—characterize the polarity of the animal. Physiological gradients, then, comprehend these physiological conditions, as well as differences in the various types of metabolism. The problem of the relation between the differentials shown here and the differentials in oxidative activity awaits further analysis.

SUMMARY

The results obtained by the desiccation of the body-wall tissue of the earthworms *H.* (= *A.*) *caliginosus* and *L. terrestris* show that in both forms a significant differential in water content exists along the long axis, being least in the anterior region and increasing toward the posterior end. In young *L. terrestris* water is equally distributed in all regions of the animal.

Live tissues from the anterior, middle, and posterior regions of the animals appropriate water when subjected to distilled water for a period of 6 hours. This appropriation is not uniform but varies with the region from which the tissue is taken. The tissue with the greatest dry weight, namely, the tissue from the anterior end, imbibes the greatest percentage of water. In *L. terrestris* more water is appropriated by the mid-region than by the posterior region; in *H. caliginosus* the posterior region appropriates more water than the mid-region.

The presence of the Ca-ion protects differentially against water appropriation. In a M/1,000 calcium chloride solution the anterior tissue gains slightly in weight, but no appreciable increase or decrease in weight is apparent in the middle and posterior tissues.

A hypotonic solution of potassium oxalate, M/1,000, increases water appropriation. The posterior pieces gain the most weight, the middle pieces less, and the anterior pieces least.

In ethyl alcohol solution—a lipoid solvent—the posterior region of *H. caliginosus* imbibes the most water, the anterior region less, and the middle region least. The anterior region of *L. terrestris* imbibes the most water, the posterior region less, and the mid-region least.

The results are interpreted as justifying an extension of the concept of physiological

gradients suggested by Huxley and de Beer (1934, p. 309). Gradients of the secondary type in annelids are apparently in some way correlated with the distribution of lipoids in the tissue, for the reaction of tissues from the anterior, middle, and posterior regions of the body to distilled water and ethyl alcohol solution, both of which have lipoid solvent properties, describe a secondary type of gradient. Primary gradients, characteristic of the adults of simple organisms and the early developmental stages of more complex organisms, appear to be associated with the distribution of water and calcium in the tissue.

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STIMULATION OF BALANUS BALANOIDES AND FUNDULUS HETEROCLITUS BY DICARBOXYLIC ACIDS[1]

(Seven figures)

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STIMULATION results from changes in the chemical or physical environment which act upon a receptor and initiate within it processes which eventuate in some response of an organism. Since processes leading to excitation are initiated at the receptor-environment interface, the structure of the interface, as well as the behavior of the stimulating agent at such a phase boundary, is most important in determining the conditions of stimulation. The rate or magnitude of excitation in a receptor unit will be a function of the disturbance in the steady state at the receptor interface. This, in turn, will be related to the concentration and configuration of the stimulating molecules.

Stimulation is related to the nature of the receptors involved, the constitution of their environment, and the properties and concentration of the stimulating agent which is added to the environment. Information as to the importance of the nature of the receptors might be obtained by comparing stimulation in different organisms, receptors of which differ chemically and morphologically. The role of the environment might be indicated by comparing chemical stimulation of a single organism in two different environments. The relationship of the number and kind of exciting molecules to stimulation might be studied by comparing stimulation by related compounds and by an investigation of it as a function of the concentration of the stimulant. In this work the first five dicarboxylic acids were used as stimulating agents on the barnacle, while the first six dicarboxylic acids and derivatives of these were used on *Fundulus*. The rate of response of the organism was studied as a function of the acid concentration. Cole and Allison (1931–32, 1932–33, 1933–34) have made a similar study on these animals, using as stimulants the fatty and mineral acids and their sodium salts.

STIMULATION OF THE BARNACLE, *Balanus balanoides* BY THE DICARBOXYLIC ACID

The barnacle is very sensitive to many different kinds of changes in its environment and responds to them by a closure of the valves or by a change in pedal rhythm. The number of regularly active animals is counted at minute intervals after the beginning of stimulation. The number of animals which have closed is expressed as a percentage

[1] This report is a portion of the thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Rutgers University, 1935. I wish to express my sincere thanks to Dr. William H. Cole and Dr. James B. Allison for their great helpfulness during this research.

[2] Contribution No. 96 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

of the total active animals at the beginning of the test. This is a measure of the effectiveness of the stimulating agent.

Cole (1931–32) found that for the sodium salts of the fatty acids, "stimulating efficiency is correlated with the potential of the anion of the acid and with the concentration of that ion near or at the receptor surface as determined by the length of the carbon chain." For 60 per cent closure or more, the effectiveness of the salts increased with length of carbon chain. For 40–50 per cent closure, formate was out of series, being more effective than propionate.

Cole and Allison (1932–33) reported that, for the fatty acids themselves, intensity of stimulation is related both to the (H^+) and to the field of force around the anion of the acid. A preliminary interpretation of the results revealed an exponential relationship between percentage of closure at the end of 4 minutes and (H^+), and showed an increasing effectiveness with length of the carbon chain of the acid. The mineral acids also fitted into this scheme, their stimulating efficiency corresponding to a fatty acid having no carbon chain at all. A further analysis of the data leads to the conclusion that the exponential relationship between percentage of closure and (H^+) is only approximate and is actually a compound sigmoid one. The three flexions in the curve are believed to be related to the possible presence of three receptor groups of different mean thresholds in the barnacle population.

EXPERIMENTAL

A stone with about eighty barnacles seated upon it was placed in a glass dish of 800 cc. capacity. The barnacles were three years of age, and all appeared to be in good condition during the tests. A different set of barnacles was used for the work done with adipic acid. About two-thirds of the animals displayed rhythmical cirripedal movement. The remainder were closed or irregularly active. Sea water was passed over the barnacles at the rate of 250 ± 15 cc. per minute at a temperature of $17° \pm 0.2°$ C. Mechanical jarring was reduced to a minimum, and the illumination was held as constant as possible. The experimental solution was passed over the barnacles for 10 minutes at the same rate of flow and temperature as the sea water which it displaced. Ample time for recovery (30 minutes) was allowed before another test was made. Two tests were made on each solution; and since they yielded similar results, averaging of the data was justified.

The acid solutions (Eastman's chemicals were used) were made up in sea water, and the solutions were allowed to stand for ½ hour before using. By this time, the (H^+), as measured by the quinhydrone electrode, had become relatively constant, and did not change noticeably during the experimental tests. As Cole and Allison have stated, "since sea water is buffered chiefly by bicarbonates, the addition of acid to it liberates H_2CO_3 and the change in pH is an approximate measure of the amount of H_2CO_3 produced when all of the acid has reacted with the bicarbonates." Over the concentration range studied, equivalent amounts of all the dicarboxylic acids will yield the same pH. This is true for all the solutions except 0.0022 N oxalic acid, which is considerably more acid than equivalent solutions of the other acids.

Eight liters of each solution were prepared. Oxalic, malonic, succinic, glutaric, and adipic acids were tested at the following concentrations: 0.0022 N, 0.0018 N, 0.0014 N, 0.0010 N, 0.0006 N, and 0.0002 N. The weakest solution was the threshold concentration of these acids for the barnacle. Percentage of closure for 0.0002 N solutions varied from 0 to about 5 per cent. Data for this concentration, while valuable in indicating

the limiting (H^+) for stimulation by the dicarboxylic acids, were not used in the graphical analysis made on the basis of the other five normalities.

RESULTS

Cole and Allison have considered that the percentage of closure 4 minutes after the experimental solution has been turned on is an adequate measure of the magnitude of response of a barnacle colony to chemical stimulation. They believed that the relative percentages of closures were independent of time and varied only with factors related to the stimulating agent. It was proposed to test this statement by making a graphical analysis of the relationship between percentage of closure and duration of stimulation by the dicarboxylic acids. Such a graphical analysis, if subject to mathematical interpretation, would lead to equations relating percentage of closure to time. The constants in such an expression would be a measure of the stimulating efficiency of a given acid concentration and would be determined by the percentage of closure at successive minute-intervals during stimulation. Such constants based on percentage of closure after all the minute-intervals would be a more reliable measure of magnitude of response, as related to acid effectiveness, than a value based on percentage of closure at the end of one particular time interval (e.g., percentage of closure after 4 minutes), since each individual count of the number of regularly active animals is subject to some variability.

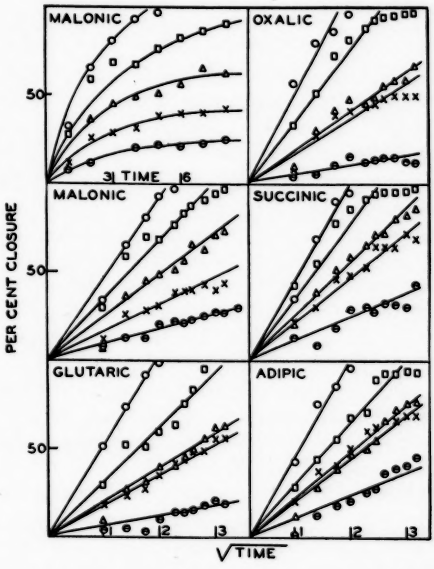

FIG. 1.—Percentage of closure as a function of the duration of stimulation in minutes. The upper left-hand plot is a typical sample of the relation obtained when percentage of closure is related to time. The remaining plots show percentage of closure related to square root of time. Solutions of different concentrations were used: 0.0022 N (○), 0.0018 N (□), 0.0014 N (△), 0.0010 N (×), 0.0006 N (⊖).

Smooth curvilinear plots are obtained for each dicarboxylic acid when percentage of closure, calculated for successive minute-intervals, is related to time which has elapsed since stimulation was begun (Fig. 1, upper left graph). The plot shown is for malonic acid. Other acids yield similar results. The curves rise from the origin with increasing steepness as the normality of the acid is raised, indicating greater effectiveness of the acid with increasing (H^+). The response to stimulation of the barnacles as measured by percentage of closure was found to increase linearly with the square root of the duration of stimulation (Fig. 1). This relationship may be represented in the form of the equation

$$\text{Percentage of closure} = Kt^{\frac{1}{2}},$$

where K is a constant representing the slope of the line when percentage of closure is plotted against $\sqrt{t}$ (K may also be obtained by determining the antilog of the y-intercept

when log percentage of closure is plotted against log t). K in the equation is obviously a measure of the stimulating efficiency of the acid, as determined by its concentration and physicochemical properties. Since the equation fits the data for stimulation by all five acids at five different concentrations, it is clear that the relationship between stimulating efficiency of different solutions does not change with duration of stimulation.

An examination of Figure 1 reveals the fact that the majority of points for percentage of closure at the end of 1 minute are lower than might be expected from the curves. This is readily explained if it is recalled that at the beginning of the test the acid concentration in the reaction chamber is zero and that it builds up to stimulating proportions only gradually. As already stated, K in the equation is a measure of effectiveness of an acid solution as determined by percentage of closure. Its magnitude is determined by the percentage of closure values for from 2 to 10 minutes, inclusive. It represents the theoretical percentage of closure at the 1-minute interval, assuming that no time is lost in building up the acid concentration flowing over the barnacles. The percentage of closure for any other time interval may be obtained by merely multiplying K by $\sqrt{t}$.

For a single acid at a definite concentration the magnitude of response by a barnacle colony will vary as the square root of the time elapsed since the beginning of stimulation. It is interesting to recall that simple diffusion in aqueous solutions also varies as the square root of time. This analogy suggests that the first step in the catenary series of events leading to the response of closure of the valves might be the diffusion of the stimulating agent through the receptor membrane before it will be in a position to upset the physicochemical equilibrium and initiate a response. Rate of diffusion is determined chiefly by the concentration gradient of the diffusing molecule, its velocity, and its chemical and physical properties. The molecular configuration would be of particular importance in determining penetration through a receptor surface. A distribution of thresholds among the barnacle population could be related to the relative time required for the stimulating agent to diffuse through the receptor surface before it reaches the receptor mechanism. Thus, those barnacles with the lowest thresholds, i.e., those which close up first when an acid is introduced into the environment, would be the ones having the thinnest or most permeable membranes on the receptor cells. The true situation may be much more complex than this, however.

K in the equation relating the percentage of closure to the square root of time is an accurate measure of the rate of response of the barnacle population to acid stimulation. How does percentage of closure, as measured by K, vary as a function of the (H^+) and with the fields of force around the anion? Figure 2 shows the K values for all the experimental solutions of the dicarboxylic acids plotted against $(H^+) \times 10^8$. A preliminary analysis indicates that an exponential relationship exists between percentage of closure, as measured by K, and (H^+). This relationship may be expressed as follows:

$$\text{Percentage of closure} = 26.45 \log (H^+) \times 10^8 - 22.$$

The exponential curve drawn through the plotted points is the theoretical curve calculated from this equation.

A careful examination of Figure 2 reveals no progressive increase in stimulating efficiency correlated with increasing length of the carbon chain of the acids. If acid stimulation is correlated with the fields of force around both the cation and anion, then it might be assumed that properties of the anion effective in stimulation are constant

for the first five dicarboxylic acids. An alternative assumption is that stimulation by the dicarboxylic acid anion is related to factors determined by the length of the carbon chain, as will be shown for *Fundulus*. The anion concentration, however, is below its threshold value for stimulation of the barnacle. That is, the barnacle is so sensitive to the H^+ which is introduced into its environment that it has already closed its valves before the anion concentration has reached stimulating proportions. If the stimulating efficiency of the cation could be reduced, it might be possible to demonstrate a differential effect related to the field of force around the anion. The stimulating efficiency of the cation can be reduced by substituting potassium in place of hydrogen on the two carboxyl

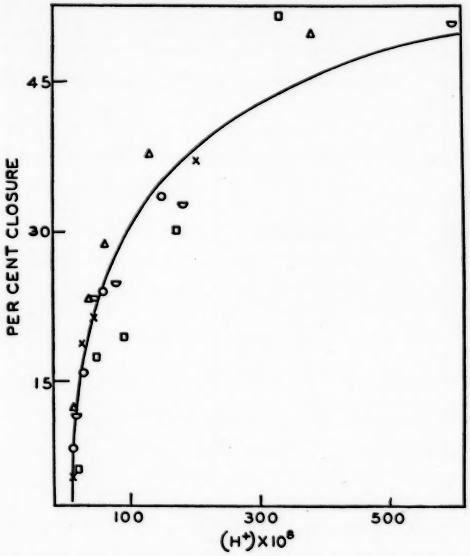

FIG. 2.—Percentage of closure after 1 minute (obtained by calculating K in the equation: percentage of closure $= Kt^{\frac{1}{3}}$) related to $(H^+) \times 10^8$. The exponential curve is the theoretical one derived from the equation: percentage of closure $= 26.5 \log (H^+) \times 10^8 - 22$. The acids used as stimulating agents on the barnacle are: oxalic (×), malonic (○), succinic (△), glutaric (□), and adipic (◡).

groups. Preliminary experiments on potassium oxalate and malonate showed very clearly that the dicarboxylic acid salts are several times less efficient as stimulating agents than the corresponding acids. Stimulation by the salts is also probably related to the field of force around the anion, since the incomplete data suggest that potassium malonate may be more effective than potassium oxalate in bringing about barnacle closure.

Cole and Allison (1932–33) reported a very definite effect in stimulation of the barnacle by the fatty acids, which was correlated with the length of the carbon chain. No similar anion effect is found here for the dicarboxylic acids. The fatty acids are famous for their ability to lower the surface tension of water and to become adsorbed at an aqueous interface. Their stimulating efficiency might be related to this tendency to be adsorbed at the receptor-solution interface. The dicarboxylic acids, because of the

greater proportion of polar groups in the molecule, would not affect the surface tension of water as greatly as the monocarboxylic acids (Langmuir, 1917). It would follow from this that they would not tend to be adsorbed so greatly at the receptor-solution interface and hence would not be so effective as stimulating agents. The surface tension of dilute solutions (0.005 and 0.1 N) of the first five dicarboxylic acids do not differ very greatly from that of distilled water (see section on *Fundulus*). The sixth acid, pimelic, has a surface tension strikingly different from the others. It would be of great interest to use pimelic acid as a stimulating agent on the barnacle and to observe whether this marked change in field of force around the anion is correlated with an increased stimulating efficiency.

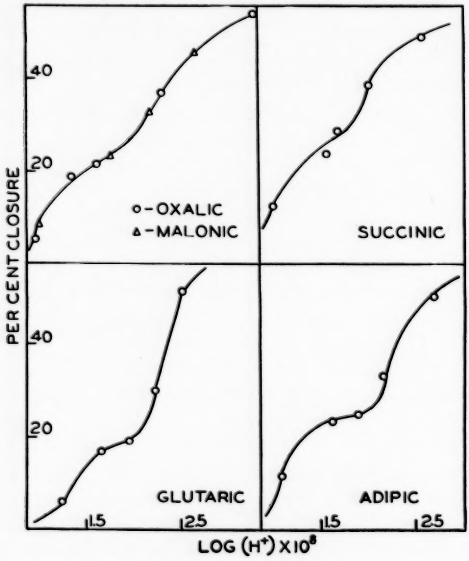

FIG. 3.—Double S-shaped curves are obtained instead of linear ones when percentage of closure is related to log $(H^+) \times 10^8$, indicating a compound sigmoid relationship for stimulation of the barnacle by these acids as a function of (H^+), rather than a simple exponential one.

Scrutiny of Figure 2 reveals the fact that the fit of the plotted points to the exponential curve is not very good. These deviations seemed to be greater than the experimental error, and a more detailed analysis of the relationship between percentage of closure and (H^+) would take these deviations into consideration and would emphasize the importance of each plotted point. When percentage of closure is related to the log $(H^+) \times 10^8$, the expected essentially linear relationship is obtained when all the points are plotted. When the data for each individual dibasic acid are treated separately, a very definite compound sigmoid relationship is obtained (Fig. 3). All the sigmoid curves are characteristically different for the several acids except for oxalic and malonic, which yield the same curve. This can only mean that stimulation by these acids is not identical, and is determined not only by the (H^+) but also by the field of force around the

anion. Thus, while stimulation by all five dicarboxylic acids is nearly the same and roughly exponential, the characteristic stimulation picture does differ for the individual acids. When all the sigmoid curves are plotted on the same axes, there is considerable overlapping. This indicates that, while the several acids yield characteristically different sigmoid relationships, their actual stimulating efficiencies are very nearly alike. Because of this overlapping of the curves the order of effectiveness will depend upon what criterion is chosen as an adequate measure of stimulating efficiency. For values of closure below 15 per cent the following order of effectiveness exists: succinic > oxalic = malonic > adipic > glutaric. If values above 60 per cent closure are considered, the order of effectiveness is: adipic > glutaric > succinic > malonic = oxalic. Still different orders would be obtained for values between 15 and 60 per cent closure. For the highest acid concentrations (i.e., those producing 60 per cent closure or more) stimulation by the dicarboxylic is dirctly correlated with properties of the anion determined by the length of the carbon chain.

Cole and Allison (1932–33) have suggested that, for a normal distribution of one kind of receptors within the population, a single S-shaped curve should describe the relationship between percentage of closure and (H^+) or log (H^+). The existence of more than one characteristic group of thresholds for the receptors should be indicated by deviations from this single sigmoid curve. It is clear from Figure 3 that each curve consists of two roughly symmetrical S-shaped portions. Following Cole and Allison's suggestion, this may indicate a bimodal distribution of thresholds among the barnacle population when stimulated by the dicarboxylic acids. These chemoreceptor groups have a differential sensitivity to the anions of these acids.

STIMULATION OF *Fundulus* BY THE DICARBOXYLIC ACIDS AND THEIR DERIVATIVES

Allison and Cole (1933–34) studied stimulation of *Fundulus* by the fatty acids in both salt and fresh water. In both environments they found that rate of response was a parabolic function of the (H^+). Stimulating efficiency increased with length of carbon chain much more markedly in salt than in fresh water. It was hoped that a study on the dicarboxylic acids would yield additional information concerning the stimulation mechanism.

PROCEDURE

The method was the same as that described previously (Sizer, 1933, 1934, 1935–36). Liquid flowed through the reaction chamber at 100 ± 5 cc. per minute and at $18° \pm 0.2°$ C. The criterion of response was a marked change in opercular rate. An ample recovery time of 2 minutes was allowed the fish. Twenty reaction times were obtained for a single acid concentration. These were given by five different fish in the fresh-water work. Experiments on one fish, however, showed that data obtained were as significant, and no more variable than, for five fish; so in the salt water tests a single animal was used throughout. The number of observations was the same for each solution, so that the percentage of probable error of the mean could be used as a measure of the variability of response.

In view of the fact that every animal is limited to a maximum rate of response determined by the receptor and neuromuscular mechanism as well as by the experimental setup, the actual reaction time was corrected by subtracting the minimum time in which the animal can respond under the given conditions. In these experiments, 4 seconds were subtracted, since in no case was a response obtained in less than 4 seconds.

STIMULATION OF *Fundulus* BY THE DICARBOXYLIC ACIDS IN SALT AND FRESH WATER

The (H^+) of the solution was found to be an adequate measure of the acid concentration which is effective in stimulation. When the (H^+) of the solution is plotted against the rate of response of the fish, a smooth curvilinear relationship appears for all the acids in either environment (Fig. 4 shows this for salt water, but curves are similar for fresh

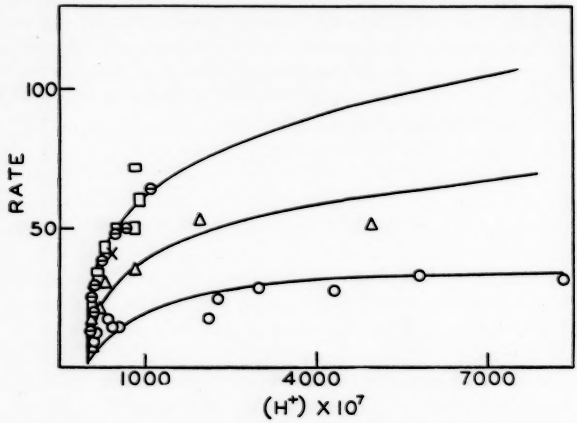

FIG. 4.—Rate of response (100/R.T. −4) of *Fundulus* to stimulation in salt water by the dicarboxylic acids plotted against (H^+). Oxalic (○), malonic (△), succinic (⊖), glutaric (□), adipic (▭), pimelic (×).

water). If the logarithm of the rate of response is related to log (H^+) (Figs. 5, 6), a linear relationship is obtained indicating a parabolic relationship between rate of reaction and (H^+). A general equation for stimulation by these acids may be set up as follows:

$$\text{Rate of reaction} = K(H^+)^n$$

where K and n are constants. The only differences among the curves for the dicarboxylic acids and their derivatives are the slope and relative positions along the y-axis. The former are measured by n, and the latter by K, in the equation. The fact that stimulation by these acids differs only by a variation of the constants in the equation indicates that the course of the receptive processes which lead to stimulation is the same for the several acids and for the two different environments, but differs quantitatively.

The foregoing equation may be expressed in terms of the corrected reaction time and the actual (H^+). The expression then becomes

$$\frac{100}{\text{R.T.} - 4} = K[(H^+) \times 10^7]^n .$$

The constants are characteristic of the fish, the environment, and the stimulating acid.

Stimulation by the various acids in fresh and salt water may be studied by considering for each acid the constants in the equation for stimulation (Table 1). An understanding

of these empirical constants would lead to a better comprehension of the various factors involved in stimulation. The constant n shows how rapidly the rate of response changes with (H^+). It may be considered a measure of the efficiency of the mechanism which relates reaction time to (H^+), and is characteristic of the receptive-process under the experimental conditions used. If it is assumed that the amount of energy change at the receptor surface is directly related to the rate of response, then an acid with a high n value will bring about this energy change more rapidly than one with a low n value.

The constant K is a relative measure of the rate of response when a threshold concentration of acid is used. Numerically it is the value of 100 times the reciprocal of the reaction time when $(H^+) = 1 \times 10^{-7}$ N. It might be regarded as a measure of the available energy of an acid molecule which plays a role in the receptive processes. Assum-

TABLE 1

CONSTANTS IN EQUATION FOR STIMULATION OF *Fundulus*

Acid	Salt Water		Fresh Water	
	K	n	K	n
Oxalic	3.8	0.24	0.09	0.73
Malonic	5.4	0.29	0.39	0.59
Succinic, etc.*	8.5	0.29	0.98	0.59
Maleic	6.5	0.32	.....	.....
Fumaric	6.5	0.22	.....	.....
l malic	8.2	0.29	.....	.....
d malic	5.7	0.29	.....	.....
i malic	4.0	0.29	.....	.....
Tartronic	3.9	0.61	.....	.....
Saccharic	15.0	0.20	.....	.....
Mucic	4.2	0.31	.....	.....
d, *l*, *m*, and *i* tartaric	10.0	0.21	.....	.....

* "Etc." refers to glutaric, adipic, and pimelic acids.

ing that a given response time is determined by a definite energy change at the receptor surface, then K would be a measure of the concentration of a substance which would be required to produce this energy change.

The importance of the receptor-solution interface has already been recognized. It was thought that there might be some correlation between the stimulating efficiency of the dicarboxylic acids and their effect upon the surface tension of water. In 0.1 N solutions of the dicarboxylic acids there is a gradual decrease in surface tension up to adipic.[3] The next higher one, pimelic, decreases the surface tension of water much more. At a concentration comparable to those used in stimulation (0.005 N) there was practically no change up to pimelic, which has a distinctly lower surface tension than water. The derivatives of these acids (0.005 N) have only very slight effects upon the surface tension of water. A comparison of stimulating efficiency with surface-tension effects reveals little correlation. While both surface tension and stimulating effectiveness are functions of the polar and nonpolar groups in the molecule, there is no close parallelism

[3] The surface tensions of these solutions were carefully measured, using the Harkins drop-weight method.

between the two. The action of the dicarboxylic acids and their derivatives at an air-water interface is not an adequate measure of the effect of these acids at the interface between solution and plasma membrane.

Adsorption is another expression of interfacial forces and may be defined as a change in concentration of a substance at an interface. The amount adsorbed, under constant conditions, varies parabolically with concentration. A similar situation is found for stimulation of *Fundulus* by acids; the rate of reaction is a parabolic function of the (H^+), which is a measure of the disturbance in the system. The orientation of an acid molecule at the receptor surface may be the first step in the series of events leading to response. Thus a substance would first have to be adsorbed at the cell-water interface in order to be effective as a stimulating agent. The ability of an acid to be adsorbed at this interface would, in part, determine its efficiency as a stimulating agent.

The stimulating efficiency of the dibasic acids increases as the carbon chain is lengthened up to succinic; the higher members—glutaric, adipic, and pimelic—bring about no further change in the energy relationships which are important in stimulation. The carbon chain may play its role in stimulation by causing the acid to be adsorbed at the receptor surface, and in this way places the H^+ in a position to upset the equilibrium between receptor and solution. This surface may become saturated by succinic, and thus further introduction of CH_2 groups would have no increased effect.

An examination of the logarithmic plots (Figs. 5–6) reveals that for a given environment all the curves, except in the case of oxalic, are parallel or have the same slope; i.e., they have identical n values in the equation relating rate of reaction to (H^+). The change in rate of reaction produced by a given alteration in equilibrium of the system as measured by (H^+) is the same for all these acids in a single environment. This is not surprising, for all the dicarboxylic acids above oxalic have the general formula, $R(COOH)_2$, where R represents the number of CH_2 groups. Oxalic differs: there is no CH_2 group in its molecule. The fact that all the dicarboxylic acids (except oxalic) yield the same slope or n value indicates that the rate of reaction of the fish may be correlated with the field of force around the nonpolar portion of the acid molecule. The H^+ plays a constant role in stimulation for all the members of the series above oxalic. Rate of response is related to the anion and increases as the carbon chain is lengthened.

The increased stimulating efficiency, as the carbon chain of the dicarboxylic acids is lengthened from 2 to 4, is expressed by increasing K values in the stimulation equation. K is considered a measure of the available energy of an acid molecule which plays a role in the receptive processes. A relationship between K and other measures of the free energy of the molecule might be expected, but such a relationship would be apparent only if both energy measures were similar functions of the total energy of the molecule. While both K and the energy of oxidation are determined by the total energy of the molecule, there is no close correlation between the two. The heat of combustion increases by 153 calories for each CH_2 group introduced into the dicarboxylic acid molecule, while K increases only up to succinic acid.

Another expression of the free energy of an acid is its dissociation constant, which is related to the polar nature of the molecule. For the dibasic acids, dissociation rapidly decreases up to succinic, beyond which there is little change. Hence a relationship may exist between K and the physical and chemical constants in so far as these may be a relative measure of the free energy of a molecule which is involved in stimulation.

A comparison of Figures 5 and 6 reveals the fact that curves for fresh-water stimula-

tion have a much steeper slope and a distinctively lower y-intercept than the corresponding curves for stimulation in salt water. The relative spread of the curves is greater for salt-water tests. Acid stimulation is parabolic in the two environments, indicating

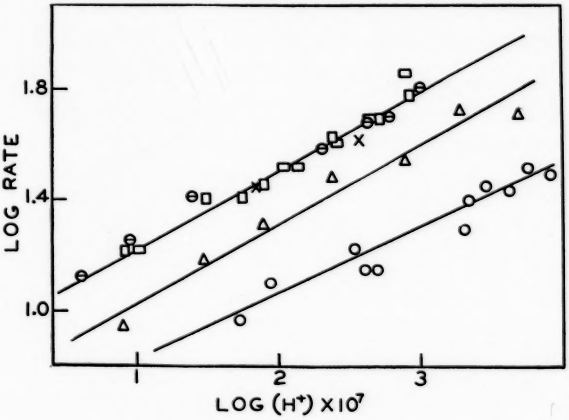

FIG. 5.—Log rate of response (100/R.T. −4) of *Fundulus* to stimulation in salt water by the dicarboxylic acids plotted against log (H^+). Symbols as in Figure 4.

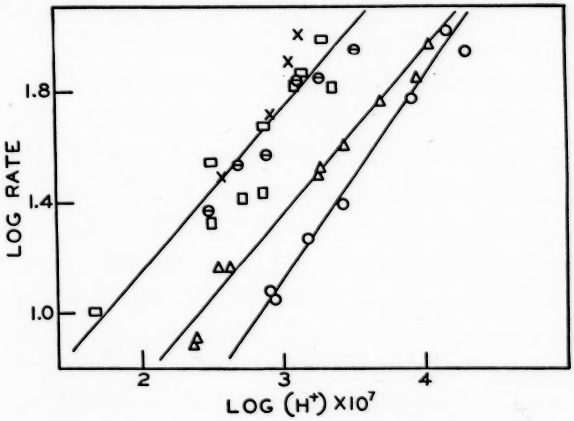

FIG. 6.—Log rate of response (100/R.T. −4) of *Fundulus* to stimulation in fresh water by the dicarboxylic acids plotted against log (H^+). Symbols as in Figure 4.

that the course of reaction in the fish is the same for both media. The receptive processes, the efficiency or velocity of which is measured by n, are characterized by a more efficient catenary series of events in fresh water than in salt. The energy available for stimulation, as measured by K, on the other hand, is much greater in salt than fresh water.

These differences may be related to the fact that sea water is a much better buffering system and would have an effect upon the orientation of acid molecules at an interface quite different from that of fresh water. The fact that K is higher in salt-water stimulation might be correlated with a greater energy available for stimulation, because a higher acid concentration is required to produce a given (H^+) in salt water than in fresh. The higher n value for fresh water might be related to the greater concentration of free undissociated acid in the fresh-water environment.

It is apparent that the constants in the equation for stimulation are determined by the particular acid used as a stimulant, are dependent upon the environment, being different for sea and fresh water, and are characteristic of the receptor-effector system of the fish.

A comparison of stimulation of *Fundulus* and the barnacle by the dicarboxylic acids reveals that the former is much more sensitive to changes in the length of the carbon chain, the latter more sensitive to alterations in the (H^+) of the solution. Stimulation in the two organisms is related to both the polar and nonpolar portions of the acid molecule. The sensitivity of the barnacle to factors measured by the (H^+) is so great that its sensitivity to changes in number of CH_2 groups is masked. In *Fundulus*, however, the effect of factors related to the (H^+) does not predominate over the effect related to the length of the carbon chain. This contrasting sensitivity is correlated with differences in the constitution and function of the receptive surfaces. The fact that stimulation in the barnacle is roughly an exponential function of the (H^+), while in *Fundulus* it is a parabolic one, should not be considered as evidence of a fundamental difference in stimulation mechanisms, however. This difference should be ascribed, rather, to the fact that in one case the percentage response of a population was the measure of effectiveness, while in the other the reaction time of a single organism was the criterion of stimulation. Similar expressions for stimulation would be expected if the method of procuring the data had been the same for the two animals.

STIMULATION OF *Fundulus* BY THE DERIVATIVES OF THE DICARBOXYLIC ACIDS IN SALT WATER

A study of the dicarboxylic acid derivatives was chiefly a comparison of certain stereo-isomers. Those used as stimulating agents were tartronic; maleic; fumaric; *d*, *l*, and *i* malic; *d*, *l*, *i*, and *m* tartaric; saccharic; and mucic acids. For these acids, rate of responses is also a parabolic function of the (H^+) (Fig. 7). The constants in the equation for each acid are presented in Table 1. Substitution of an OH group or a double bond into a dicarboxylic acid molecule changes its effectiveness in stimulating *Fundulus*. In general, K, in the equations for stimulation, is decreased by such substitution for the compounds studied (tartaric and saccharic acids are exceptions), but the slope of the stimulation curve, represented by n, may be altered in either direction.

It may be stated that in general those isomers having very similar physical and chemical properties have the same constants in the equation, while those of widely different properties yield different values in the expression for stimulation. The more chemically active member of an isomeric pair is, in general, the more efficient stimulating agent. For closely related isomeric compounds the relationship between the energy of a molecule involved in stimulation and the energy of a molecule expressed as heat of oxidation may prove useful. Maleic and fumaric acids have the same K value in their equation for stimulation. They also have almost identical values for heat of combustion. N in the equation was considered a measure of the efficiency or velocity of the receptive proc-

esses involved in stimulation. Maleic has a higher solubility, has less of an effect upon the surface tension of water, is a stronger acid, and has the higher chemical activity of the two. It also has a higher n value in the equation.

The heats of combustion of d, l, and i malic cannot be compared from the literature at hand. They have widely different K values in the stimulation equation. In so far as heat of combustion may be an expression of the type of energy involved in stimulation, a relationship between the two might be expected. As shown previously, heat of combustion is an inadequate measure of the energy of a molecule involved in stimulation, and there might not be any apparent correlation here. All three acids, however, have nearly identical physical and chemical properties, and the rate of change of reaction time with (H^+) (as expressed by n) is the same for these isomers.

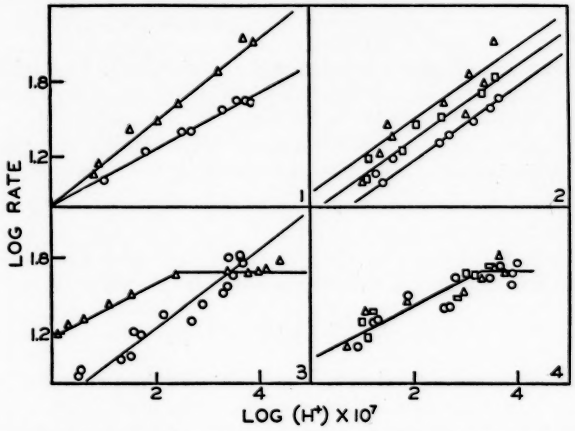

FIG. 7.—Log rate of response (100/R.T. −4) of *Fundulus* to stimulation in salt water by certain derivatives of the dicarboxylic acids plotted against log (H^+).

1. Malonic (△), fumaric (○).
2. *l* malic (△), *d* malic (□), *i* malic (○).
3. Saccharic (△), mucic (○).
4. *l* tartaric (▭), *m* tartaric (△), *i* tartaric (□), *d* tartaric (○).

D, *l*, *m*, and *i* tartaric have practically the same heat of combustion and very similar physical and chemical properties. The three isomeric acids and the racemic mixture of *d* and *l* all give the same constants in the equation. The rate of response does not change with (H^+) below a pH of 3.7.

A comparison of the isomers, saccharic and mucic acids, reveals that saccharic has a much higher heat of combustion and also has a much higher K value than mucic. Its dissociation constant is slightly higher and its solubility much greater, but its value for n is lower than for mucic. The actual reaction time of the fish is a function of both K and n, and over the lower concentration range saccharic acid is the more efficient stimulating agent. An increase in acidity by saccharic beyond pH 4.6 brings about no further decrease in reaction time.

If the receptors involved in stimulation have different thresholds, then those with

the highest thresholds are not stimulated by tartaric or saccharic acids. Possibly at high concentrations these acid molecules are not oriented in such a manner as to upset the equilibrium at the cell-solution interface and thus introduce new units which might accelerate the receptor-response mechanism. The break in the tartaric and saccharic acid curves indicates a change in the mechanism of reaction. This would be predicted for the other acids if they could only be studied at sufficiently high concentrations. Only within the limits studied, does a parabolic relationship exist between rate of reaction and (H^+). There is no justification for extending the theoretical curves, for beyond the experimental limits the mechanism of reaction may be totally different.

The probable error of the mean reaction time

$$\text{P.E.} = \pm .8453\ \Sigma \frac{(+v)}{n\sqrt{n-1}}$$

was calculated for every acid concentration used as a stimulant. Probable error decreases with (H^+) or increases with reaction time. Variability of response or probable error expressed as a percentage of the corrected reaction time (100 P.E./R.T. $-$ 4) is essentially constant (about 3.55 per cent) for a given acid and does not vary with (H^+) or response time. This indicates that the mechanism of reaction does not change with concentration.

SUMMARY

1. Stimulation of the barnacle by the dicarboxylic acids reveals a linear relationship between percentage of closure and the square root of the duration of stimulation according to the equation

$$\text{Percentage of closure} = Kt^{\frac{1}{2}},$$

where K is a relative measure of the effectiveness of the acid used. Certain analogies between percentage of closure and diffusion as functions of time are apparent. A preliminary analysis indicates that an exponential relationship exists between percentage of closure and (H^+):

$$\text{Percentage of closure} = 26.5 \log (H^+) \times 10^8 - 22.$$

There is no progressive increase in stimulating efficiency correlated with the addition of CH_2 groups to the acid molecule. A more detailed analysis of the data reveals that this exponential relationship is actually a compound sigmoid one. The curve for each acid is composed of two symmetrical S-shaped portions and is characteristically different from the curves for the other dibasic acids (oxalic and malonic give the same curve, however), indicating a differential sensitivity to the field of force around the anion.

2. Stimulation of *Fundulus* by the dicarboxylic acids in salt or fresh water, or by the derivatives of these acids in salt water, is characterized by a parabolic relationship between rate of response and (H^+) of the type

$$\frac{100}{\text{R.T.} - 4} = K[(H^+) \times 10^7]^n.$$

The constants in the equation are characteristic of the fish, the stimulating acid, and the environment. The constant n is a measure of how rapidly the rate of response

changes with (H^+), while K is a relative measure of the rate of response when a threshold concentration of acid is used. Stimulation by all the dicarboxylic acids above oxalic yields the same n value. K increases exponentially as the number of CH_2 groups in the molecule is increased from 0 to 2. Beyond succinic there is no further increase. N is lower, and K is higher, for stimulation in salt water by the dibasic acids as compared with fresh water. This emphasizes the importance of both environment and the receptor mechanism in determining stimulation.

3. Stimulation of *Fundulus* by the derivatives of the dicarboxylic acids in sea water is characteristically different from that of the corresponding unsubstituted acids. A comparison of certain isomers among these derivatives indicates that the isomers of similar chemical and physical properties have the same stimulating efficiency, but those with different properties were not equally effective.

4. The barnacle is more sensitive than *Fundulus* to the (H^+), while *Fundulus* is more sensitive to the anion. These differences are related to the specific chemical and morphological properties of the receptor mechanism.

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THE EFFECT OF DIFFERENT RELATIVE HUMIDITIES ON RESPIRATORY METABOLISM AND SURVIVAL OF THE GRASSHOPPER CHORTOPHAGA VIRIDIFASCIATA DE GEER

(Two figures)

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BODINE (1921) showed that the hibernating nymphs of *Chortophaga viridifasciata* are able to absorb water from the air when placed in sealed jars containing wet sand, and that they lose water when placed in calcium chloride desiccators. He found that, when starved at 0°–9° C., they live about 2 weeks; at 23°, they live 1 week; and at 38°, only 3 or 4 days. He stated that "starvation in the grasshopper results in a rapid loss in water which has a decidedly quick and fatal effect." He also showed that starvation causes a decrease in respiratory metabolism as measured by carbon dioxide production.

Caldwell (1925) found that when larvae of *Tenebrio molitor* were desiccated to less than one-half of the possible vital limit there was an increase in carbon dioxide production. Further desiccation resulted in a decreased carbon dioxide production which fell below normal before the vital limit was reached. The same author, using the eight-spotted forester moth, *Alypia octomaculata*, obtained a decreased carbon dioxide production in all stages of desiccation. Bodine (1933) observed that subjecting the developing eggs of the grasshopper, *Melanoplus differentialis*, to hypertonic solutions resulted in a decreased rate of oxygen consumption.

Since *C. viridifasciata* absorbs water in moist air and loses it in dry air, it was decided to starve individuals at different relative humidities to determine the degree of saturation of the air at which the water content of the insect remains approximately constant. The time of survival and the water content at the time of death were recorded for each humidity. To determine the effect of water content on metabolic rate, readings on respiratory exchange were made on starved individuals kept under conditions which resulted in an increased water content, a constant water content, and a rapid desiccation.

MATERIAL AND METHODS

Third, fourth, and fifth instar nymphs and adults were used in these experiments. The nymphs were collected during the fall, winter, and spring months as needed; and the adults were reared from these nymphs. They were kept in the laboratory in large breeding-cages and were continuously supplied with fresh lettuce, except during the period of experimentation, when they were starved.

In the humidity experiments each individual was weighed on a chainomatic balance, sensitive to 0.1 mg., and placed in a vial stoppered with cotton, which was then kept at the proper humidity. Temperature was controlled near 25° C. Daily weight readings were made until death, when residual water content was determined by desiccation at 50°–60° C. to a constant weight. Fecal material was included in all weight readings. Normal water content was determined on ten to twenty individuals from each group of grasshoppers used in these experiments.

The desired humidities were obtained by using saturated solutions of various salts in the bases of ordinary desiccators. The relative humidity values for these solutions were calculated from the data of Sweetman (1933) and the *International Critical Tables*.

Respiratory metabolism was measured with the modified Krogh manometer (Bodine and Orr, 1925). During the experiments the manometers were placed in a water bath kept at a constant temperature of 25° C. For the determination of oxygen consumption, 2 cc. of 2 per cent KOH were placed in each manometer flask to absorb the carbon dioxide. Readings on oxygen consumption were made over a period of several hours. The KOH was then removed, and the flasks were washed thoroughly. The manometers were then returned to the water bath, and readings were again made over a period of several hours. From the difference between the first and second readings, the carbon dioxide production and respiratory quotients were determined. The grasshoppers used for the metabolism readings were kept in the humidifiers at the various humidities except

TABLE 1

RELATION BETWEEN THE ORIGINAL WATER CONTENT AND THE PERCENTAGE OF WEIGHT LOST AT THE TIME OF DEATH OF *Chortophaga viridifasciata* KEPT AT 0–5 PER CENT HUMIDITY

No. of Individuals	Stage of Development	Original Water Content (per Cent)	Water Content at Time of Death (per Cent)	Weight at Time of Death as Percentage of Original Weight
11	Hibernating nymphs	65.6	56.1	72.5
8	Hibernating nymphs	68.1	58.8	72.4
13	Growing nymphs	70.7	56.3	61.1
13	Growing nymphs and adults	73.6	57.9	54.8
20	Growing nymphs and adults	75.9	58.8	56.2

for the daily period of 4 or 5 hours required for the respiratory experiment. This daily exposure to the humidity of the manometer flask probably did not materially change the water content of the tissues, since complete desiccation at the time of death showed the same residual water as in comparable series not used in respiratory experiments.

OBSERVATIONS

A seasonal variation in the normal water content of *Chortophaga* nymphs was observed. The average water content of those collected October 22, 1933, was 68.1 per cent, while those collected November 26, 1933, had 65.6 per cent water and those collected the following April had 75.9 per cent water. It was also observed that nymphs, collected in the winter and kept at room temperature, began to grow and their water content increased to a value comparable to that of growing grasshoppers collected in the field.

Table 1 shows that the average fatal limit of desiccation of grasshoppers starved at 0–5 per cent humidity is the same (56.1–58.8 per cent) regardless of the original water content or the stage of development. If the original water content is low, they can survive a loss of only 28 per cent of their original weight; while if it is high, they can lose more than 40 per cent of their original weight before death occurs.

Table 2 contains the calculated and observed water contents at death and the time of survival at each humidity. The calculated water content was obtained by assuming that all of the weight lost was the result of a loss of water. In this experiment, growing (fourth and fifth instar) nymphs and adults were used. No differences in the time of survival or the water content at death of nymphs and adults were observed, and hence the results are averaged together in this table. In every case the calculated water contents at death are considerably lower than the observed values. At 82 per cent humidity, water content remained approximately constant during starvation. At higher humidities it increased; and at lower humidities it decreased. Between the humidities of 27 and 76 per cent the water contents at death were maintained at relatively high values (68.1–70.0 per cent); and at 0–5 per cent the tissues were rapidly

TABLE 2

WATER CONTENT AND TIME OF SURVIVAL OF *Chortophaga viridifasciata* NYMPHS AND ADULTS STARVED AT DIFFERENT RELATIVE HUMIDITIES
(Normal water content = 75.9 per cent)

No. of Grasshoppers Used	Salt Used (Saturated)	Approximate Humidity (per Cent)	Water Content at Death		Days Survived
			Calculated (per Cent)	Observed (per Cent)	
15	H_2O	96	74.3	82.4	6.6
10	K_2SO_4	92	73.3	78.4	5.6
10	$ZnSO_4$	82	71.0	76.2	5.5
10	KBr	78	67.2	72.0	5.5
10	NaCl	76	66.0	70.0	5.3
15	$NaNO_2$	61	63.2	69.7	6.4
15	NaBr	56	65.9	68.1	5.2
15	$CaCl_2$	31	60.4	68.7	6.5
15	$KC_2H_3O_2$	27	60.7	68.4	6.2
15	LiCl	12	60.4	66.5	5.2
20	Dry $CaCl_2$	0–5	56.8	58.8	6.6

desiccated. Even at a humidity as low as 12 per cent, the water content at death was higher than that of normal hibernating nymphs (see Table 1). These facts, together with the observation of Bodine (1923) that hibernating nymphs must feed at intervals if they are to survive the winter, suggest that at all humidities, except possibly at 0–5 per cent, death was caused by starvation and not by desiccation.

In Figure 1 are plotted the changes in weight which occurred during starvation at different humidities. In general the rate of weight loss is inversely proportional to the relative humidity. However, the differences in the rates of weight loss are very small at humidities between 27 and 76 per cent, the rate curves frequently crossing each other. During the first 24 hours at 96 per cent, there was a rapid increase in weight as a result of the absorption of water. This initial increase was then followed by a gradual decrease in weight until, at the time of death, the animals weighed less than at the start of the experiment. Slight initial increases in weight also occurred at humidities of 82 and 92 per cent.

In Figure 2 the percentages of the original weight lost during different periods of starvation are plotted against the saturation deficiency. An examination of this figure shows that weight loss is not directly proportional to the saturation deficiency except at high saturation deficiencies (low humidities). Below 5.7 mm. of mercury (76 per cent relative humidity at 25° C.) there is a rapid decrease in the rate of weight loss as the saturation deficiency is decreased. The normal water content of the grasshopper nymphs and adults used in these experiments was 75.9 per cent, suggesting that, in those forms which are hygroscopic, loss of water is hindered when its concentration in the air is

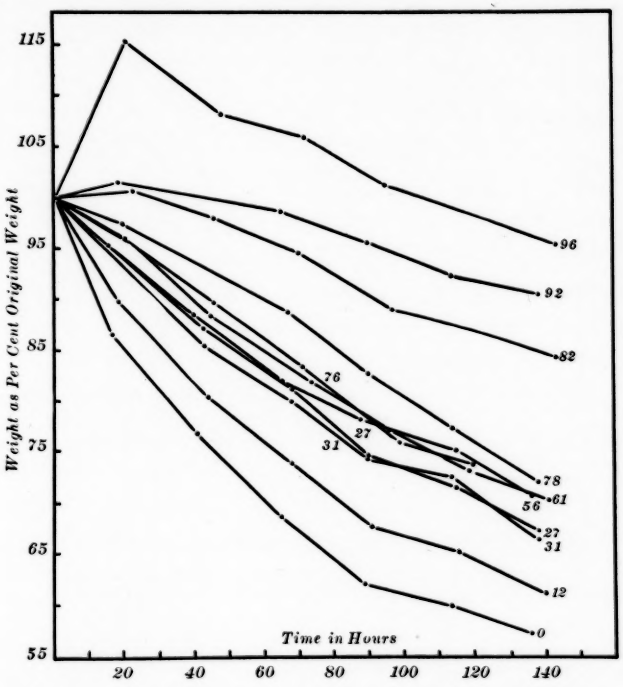

FIG. 1.—Rate of weight loss of *Chortophaga viridifasciata* nymphs and adults at different relative humidities. Numbers indicate relative humidities.

greater than in the tissues. However, the losses in weight at saturation deficiencies between 10.45 and 5.32 mm. of mercury are much greater than would be expected if weight loss were directly dependent on the saturation deficiency. Mellanby (1932*a*) states that, if loss of weight is directly proportional to the saturation deficiency, the expression $\frac{\text{Loss in weight}}{\text{Saturation deficiency}}$ should be constant. Table 3 contains a tabulation of these ratios for different periods of starvation. After 20 hours of starvation, they are constant for all saturation deficiencies between 23.75 and 5.32 mm. of mercury. Below 5.32, weight actually increased during the first 20 hours. However, for starvation

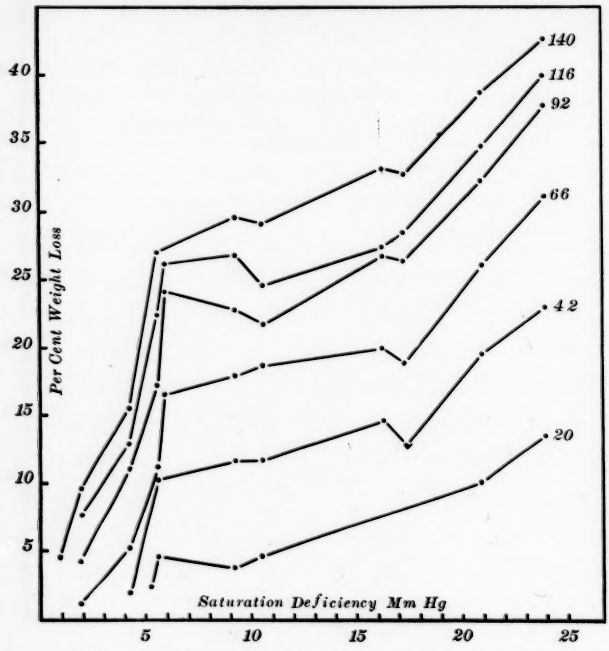

FIG. 2.—Rate of weight loss at different saturation deficiencies. Numbers indicate the duration of the starvation period in hours.

TABLE 3

VALUE OF THE RATIO $\frac{\text{LOSS IN WEIGHT}}{\text{SATURATION DEFICIENCY}^*}$ FOR DIFFERENT PERIODS OF STARVATION AT 25° C.

APPROXIMATE HUMIDITY (PER CENT)	SATURATION DEFICIENCY (IN MILLIGRAMS OF MERCURY)	DURATION OF STARVATION PERIOD					
		20 Hours	42 Hours	66 Hours	92 Hours	116 Hours	140 Hours
0	23.75	0.56	0.97	1.31	1.59	1.68	1.79
12	20.9	0.53	0.93	1.24	1.55	1.67	1.85
27	17.34	.....	0.73	1.09	1.45	1.64	1.89
31	16.39	.....	0.88	1.22	1.57	1.67	2.03
56	10.45	0.45	1.11	1.72	2.08	2.38	2.79
61	9.26	0.42	1.14	1.94	2.47	2.94	3.20
76	5.70	0.80	2.00	2.89	4.24	4.59	.....
78	5.32	0.47	.....	2.10	3.21	4.23	5.26
82	4.27	.....	0.46	1.21	2.57	3.04	3.63
92	1.90	.....	.....	0.63	3.26	4.05	5.05
96	0.95	.....	.....	.....	.....	.....	4.73

* Saturation deficiency is the pressure of the water vapor which must be added to a sample of air to saturate it, without altering the temperature.

periods of 42 hours or longer, the ratios are constant only for saturation deficiencies between 23.75 and 10.45 mm. of mercury.

In Table 4 are tabulated the averages (with their probable errors) of the respiratory readings made on grasshoppers subjected to humidities of 0–5, 82, and 96 per cent. Female grasshoppers are very much larger than males of the same stage of development

TABLE 4

EFFECTS OF HUMIDITY ON THE RESPIRATORY EXCHANGE OF STARVED *Chortophaga viridifasciata* NYMPHS AND ADULTS

Hours in Humidifier	No. of Individuals	O_2 Mm.^{3}/Min./ Grasshopper	O_2 Mm.^{3}/Min./ Gram	CO_2 Mm.^{3}/Min./ Gram	R.Q.
	$CaCl_2$, 0–5 per Cent Relative Humidity				
0	25	2.66±0.15	11.14±0.31	9.76±0.34	0.876±0.008
24	23	2.05±0.11	10.86±0.48	8.65±0.48	0.796±0.010
48	25	2.08±0.13	10.06±0.32	7.87±0.29	0.782±0.010
72	26	1.60±0.10	8.80±0.35	6.72±0.31	0.764±0.008
96	26	1.56±0.11	8.98±0.22	6.92±0.24	0.771±0.011
120	22	1.44±0.10	8.58±0.38	6.44±0.33	0.751±0.014
	$ZnSO_4$, 82 per Cent Relative Humidity				
0	25	2.99±0.12	12.88±0.52	10.91±0.50	0.847±0.009
24	23	2.42±0.10	10.60±0.47	8.64±0.49	0.814±0.014
48	23	2.08±0.12	9.42±0.44	7.39±0.48	0.785±0.007
72	23	1.95±0.11	9.38±0.55	7.52±0.47	0.801±0.009
96	21	2.00±0.11	9.83±0.57	7.78±0.51	0.791±0.008
120	21	1.84±0.11	9.11±0.50	7.21±0.40	0.791±0.006
	H_2O, 96 per Cent Relative Humidity				
0	25	2.83±0.16	12.22±0.48	10.55±0.47	0.863±0.009
24	24	2.16±0.14	9.71±0.46	7.96±0.39	0.820±0.012
48	25	2.20±0.15	9.92±0.46	7.81±0.43	0.787±0.012
72	24	1.97±0.15	9.54±0.41	7.50±0.34	0.786±0.007
96	23	1.87±0.14	8.25±0.38	6.45±0.32	0.782±0.009
120	20	1.92±0.14	8.92±0.56	7.00±0.46	0.785±0.009

and usually have a much higher metabolic rate, as measured per individual. However, since the larger animal has a smaller area of respiratory surfaces per unit of weight, the metabolic rate of the female grasshopper is lower than that of the male, when measured per gram body weight. No differences were noted in the metabolic rate of nymphs and adults of the same body weight. In this experiment, approximately the same number of nymphs and of male and female adults was used at each humidity. The oxygen consumption per minute per grasshopper was calculated, as well as that per gram body weight, since during starvation, particularly at 0–5 per cent humidity, each grasshopper loses weight very rapidly. At each humidity, starvation results in a decrease in oxygen

consumption, carbon dioxide production, and respiratory quotient. At 0–5 per cent humidity the rate of oxygen consumption after 5 days of starvation was 54.1 per cent of its original value. At 82 per cent humidity it was 61.5 per cent; and at 96 per cent humidity it was 67.8 per cent of the original rate. The figures contained in Table 2 show that at 0–5 per cent the tissues were dehydrating, at 82 per cent their water content remained constant, and at 96 per cent it increased. Hence, there is a slight decrease in respiratory rate with a decrease in the water content of the tissues. However, the variations existing within each series are so great that the differences obtained at different humidities are not statistically significant. As indicated in Table 5, the ratios of the difference to the probable error of the difference never equals 3, even after 120 hours of starvation. At each humidity, regardless of the water content of the tissues, the decrease

TABLE 5

PROBABLE SIGNIFICANCE OF DIFFERENT RESPIRATORY READINGS OBTAINED AFTER 120 HOURS OF STARVATION AT DIFFERENT HUMIDITIES

Readings Compared	Differences between Means	Probable Error of the Difference	Difference/ Probable Error of the Difference
O_2 mm.^{3}/min./grasshopper:			
0 and 96 per cent relative humidity..	0.48	0.172	2.2
0 and 82 per cent relative humidity..	0.40	0.148	2.7
O_2 mm.^{3}/min./gram:			
0 and 96 per cent relative humidity..	0.34	0.67	0.5
0 and 82 per cent relative humidity..	0.53	0.62	0.8
Respiratory quotient:			
0 and 96 per cent relative humidity..	0.034	0.016	2.1
0 and 82 per cent relative humidity..	0.040	0.015	2.6

in respiratory exchange and respiratory quotient must therefore be largely the result of starvation.

DISCUSSION

The seasonal changes observed in the water content of grasshopper nymphs are almost identical with those reported by Bodine (1921 and 1923). He observed that hibernating nymphs, when brought into the laboratory and subjected to favorable temperatures, absorb water and begin growing. In Bodine's experiments the water content of growing nymphs was 75 per cent; in the present experiments it was 73.6 per cent in one group of nymphs and 75.9 per cent in another group. Bodine found that nymphs, starved at 23° C., lived about 1 week and that the maximum loss of weight at death ranged from 20 to 25 per cent. In the present work it was found that at 25° C. both nymphs and adults lived an average of 5.2–6.6 days, regardless of humidity. However, the maximum amount of weight lost at death depended upon the humidity and the initial water content. In dry air, grasshoppers with a high initial water content lost more than 40 per cent of their weight before death occurred. At high humidities, death occurred with very little loss of weight; and at 96 per cent humidity, the weight at death

was more than 90 per cent of the initial weight. Bodine believed that starvation results in a rapid loss of water, which in turn causes death. However, it has been demonstrated that, at high humidities, death by starvation occurs just as soon as at low humidities, although at the higher values no lowering of the water content was observed.

The results just described for grasshopper nymphs and adults are very different from those reported for Japanese beetle larvae, prepupae, and pupae by Ludwig and Landsman (1937). The time of survival of the grasshopper was the same regardless of humidity, and the observed water content at death remained above the normal water content of hibernating nymphs at humidities of 12 per cent and above. In Japanese beetle larvae, prepupae, and pupae, the time of survival varied directly with the humidity; and the water content at death, except at the higher humidities, was constant for each stage. Grasshoppers were able to absorb water from the air at high humidities; whereas no absorption of water was observed in the Japanese beetle. Grasshopper nymphs and adults died of starvation, except in dry air; while Japanese beetle larvae died of desiccation, except in saturated air. In the latter form the prepupa and pupa metamorphose at high humidities.

In grasshopper nymphs and adults, the observed water content at death was always higher than the calculated value; whereas in the various developmental stages of the Japanese beetle, the loss of weight was due entirely to a loss of water, and the calculated and observed water contents at death were the same, except in saturated air. This difference probably indicates a rapid metabolism of tissue solids in the grasshopper, possibly with the retention of some metabolic water; while in the Japanese beetle, very little tissue was utilized during starvation, the individuals dying of desiccation. The fact that grasshoppers died in less than 1 week of starvation, while Japanese beetle larvae lived nearly a month at high humidities, is further evidence of a difference in metabolic rate. The fact that grasshoppers maintained a high water content at humidities as low as 12 per cent is evidence for the conservation of metabolic water at low humidities. Mellanby (1932*a* and 1932*b*) found a conservation of metabolic water in the meal worm, *T. molitor*, and in the bedbug, *Cimex lectularius*. On the other hand, Evans (1935) found that in the prepupae and pupae of the blowfly, *Lucilia sericata*, the loss of dry matter is constant at all humidities, and the greater loss of weight at low humidities is due entirely to a greater loss of water. Similarly, MacLeod (1935) observed that the tick, *Ixodes ricinus*, is not able to conserve water, the loss of weight being a direct indication of the evaporation of water from the body.

In the present experiments it was found that the loss of weight of *Chortophaga* nymphs and adults is not proportional to the saturation deficiency. Buxton (1932*a*) plotted some of the results of Bodine (1921), which indicated that in hibernating *Chortophaga* nymphs this relationship is linear. Buxton utilized data obtained at different temperatures in dry air and in saturated air but did not have available data for intermediate humidities. Figure 2 and Table 3 show that at 25° C. the loss of weight at low humidities is in agreement with this conclusion of Buxton, being proportional to the saturation deficiency. However, because of the ability of this form to absorb water from the air at high humidities, the relationship does not hold for all of the values studied. Furthermore, at saturation deficiencies between 10.45 and 5.32 mm. of mercury, the loss of weight is greater than calculated on the basis of the deficiency law. Table 2 shows that, in spite of this loss in weight, the water content remains high and at a constant value. These observations suggest the greater utilization of tissue solids at higher humidities and the retention

of metabolic water at lower humidities. A similar condition was described by Buxton (1932*b*) for the bug *Rhodnius prolixus*. He found that after 42 days of starvation at 30, 60, and 90 per cent humidity the loss of weight was not affected by atmospheric humidity. Buxton found, however, that more solid material was oxidized at 90 per cent humidity than at 60 per cent, and more at 60 per cent than at 30 per cent.

The normal rate of respiratory exchange reported in the present experiments is in conformity with the results of Kleinman (1934), using the same species of grasshopper. The decrease in respiratory rate during starvation is comparable to that described by Bodine (1921) for the grasshopper, *M. differentialis*. It is interesting to note that Caldwell (1925) found an increased carbon dioxide production during the early stages of desiccation in starved meal worms, as compared with control worms which were starved but not desiccated. However, in both desiccated and control worms, starvation resulted in a decreased carbon dioxide production, as compared with controls which were fed. Caldwell suggested that in some animals desiccation stimulates physiological activity. No significant change in the respiratory rate of *Chortophaga* resulted from desiccation. The observed decrease in the respiratory quotient suggests the utilization of stored fats during periods of starvation.

SUMMARY

1. Regardless of the initial water content, *C. viridifasciata* nymphs and adults can survive desiccation until their water content averages 56.1–58.8 per cent of the body weight. If the original water content is low, death results when only 28 per cent of the original weight has been lost; while if it is high, they can survive a loss of more than 40 per cent of the original weight.

2. The time of survival of starved nymphs and adults is the same (5.2–6.6 days) regardless of humidity.

3. Individuals starved at high humidities absorb water from the air, so that at the time of death their water content is actually higher than originally. Starvation at 82 per cent humidity produces no change in water content; and below this value, water content is decreased.

4. During starvation at humidities between 27 and 76 per cent water content is maintained at a constant high value, averaging 68.1–70.0 per cent of body weight. This high water content at low humidities may result from the retention of metabolic water.

5. Loss of weight is not proportional to saturation deficiency except at low humidities. The relationship is complicated by the absorption of water at high humidities.

6. Starvation results in a decrease in the respiratory exchange and respiratory quotient. Oxygen consumption of grasshoppers storved at 0–5 per cent humidity decreased to 54.1 per cent of the original value. At 82 per cent humidity, it decreased to 61.5 per cent; and at 96 per cent humidity, to 67.8 per cent of the original value. However, the variations existing within each series are so great that these differences in the rate of respiratory exchange are not statistically significant.

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THE SHEDDING REACTION IN ARBACIA PUNCTULATA[1]

(One figure)

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IT HAS been the custom for twenty-five years and perhaps longer to obtain eggs and sperm from the sea urchin by cutting horizontally through the test or by removing the lantern. If animals cut in this way are placed aboral side down in a watch glass, the eggs or sperm are at once extruded through the five genital pores. No one has investigated the question as to just why injury invariably results in the shedding of the genital products. In view of recent developments in the field of humoral physiology, it is of considerable interest to determine whether this specific reaction is of humoral or neural origin. The object of the present investigation is to determine the nature of the stimulation which causes sea urchins to spawn when injured. Solution of this problem might indicate possible relationships to invertebrate stimulation in general.

FACTORS ASSOCIATED WITH SPAWNING IN INVERTEBRATES

An accurate analysis of the physiological factors associated with spawning in animals is often impossible due to the complicated organs and reflexes involved. In a number of marine invertebrates, however, these processes are less complicated and it has been possible to determine the factors responsible for the initiation and continuation of the shedding process. Orton (1920) and Galtsoff (1930) found temperature changes associated with spawning periods in *Ostrea* as did Elmhirst (1922), Orton (1929), and Moore (1934) for *Echinus*. Ohshima (1925) has found that light is a factor in the shedding of *Thyone*. The effect of lunar periodicity is reviewed by Fox (1922, 1923*a*), who gives an extensive bibliography. Fox (1923*b*), Lillie and Just (1913), and Galtsoff (1930, 1932, 1934) demonstrated chemical stimulation of shedding.

Environmental factors are ineffective in stimulating shedding in *Arbacia punctulata*, for a series of preliminary experiments similar to those of Tennent, Gardiner and Smith (1931) and Fox (1923*b*) gave negative results. There is no correlation between the presence of spawning individuals or of the genital products of either sex with the onset of spawning. Artificially raising the temperature of the water will often initiate shedding in ripe urchins. This is, however, not invariable and is often associated with death. Raising the carbon dioxide content or lowering the oxygen tension of the water did not induce spawning. There is no high incidence of spawning at the stages of the moon, so that lunar periodicity plays no important part. The shedding of *Arbacia*, then, can have no explanation based on experimental evidence derived from other species or from any known normal variation within its own environment.

MATERIAL AND METHODS

The work was done at the Woods Hole Marine Biological Laboratory during the summer of 1935 under the direction of Dr. L. V. Heilbrunn, to whom thanks are due for the

[1] A thesis in zoölogy presented to the faculty of the Graduate School, University of Pennsylvania, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

suggestion of the problem and with whose kind help and advice the work was carried out. The histological study was made at Wellesley College. The general plan was very simple. Urchins collected in Woods Hole were used. After injury or treatment with stimulating substances, the animals were placed in finger bowls holding approximately 250 cc. of sea water and were observed individually. In some of the immersion experiments as many as 8 or 10 animals were treated at the same time in 1,500–2,000 cc. of sea water. The injections were made with a glass pipette marked to deliver 1 cc. of solution and the same amount was always used regardless of the concentration. All the experiments were done at room temperature, and no attempt was made to control the pH except in experiments where specific H-ion concentrations were studied. In that case adjustments were made with 0.1 normal HCl and the sea water was allowed to stand overnight to avoid changes due to the carbonates. The H-ion concentrations were determined colormetrically with apparatus from the La Motte Chemical Products Company.

The isotonic solutions were made with C.P. salts in the following concentrations, considered isotonic with sea water based on freezing-point determinations:

$CaCl_2$	0.38 molar	KCl	0.57 molar
$MgCl_2$	0.37	NaCl	0.56
$MgSO_4$	0.97		

Concentrations isotonic with sea-urchin eggs, as determined directly by volume measurements of the egg, are somewhat lower than these figures. These concentrations—0.3 molar for calcium and magnesium and 0.53 molar for potassium and sodium—have exactly the same effects on the gonads as the foregoing concentrations, except that they would naturally not be so effective in the greater dilutions.

The time intervals before shedding were determined by observation, the end point being the first appearance of the spawn. They cannot, therefore, be absolute latent periods; but since the averages of each type of stimulation were taken for from 25 to 50 animals (more in some cases), the comparative value is immediately apparent.

MORPHOLOGY OF THE GENITAL SYSTEM OF *Arbacia*

Before an analysis can be made of the shedding reaction, it is essential to understand the morphology of the reproductive mechanism involved. Early references to echinoderm anatomy include Agassiz (1872), Romanes (1885), Fredericq (1876), and Hammann (1889). Recent papers on the sea urchin have, in general, referred back to these papers. For the purposes of this investigation the muscular and nervous systems in their relationship to the generative organs are of particular interest.

The five gonads of *A. punctulata* are arborescent glands suspended by mesenteric folds from the interambulacral plates in the apical half of the body cavity. Each of the organs opens to the exterior through a genital duct which passes through the aboral circular sinus to a genital pore in the basal plate of the apical system. Externally it is generally considered impossible to distinguish the males from the females. Internal observation of sexually mature males shows the gonads to be milk white or creamy while the ovaries are reddish or orange brown in color. Spawning is the extrusion of the genital products by the contraction of the gonad wall, and there are no accessory structures associated with the pores or ducts.

Hammann's (1889) figure of the ovary of *Toxopneustes* shows the wall of the ovary

to be composed of an outer epithelial layer, a connective tissue layer, and an inner layer of follicle cells and oöcytes. In the description, however, he says that a cross-section shows in addition a layer of muscle cells. Tennent, Gardner, and Smith (1931), who made a cytological study of the gonads of *Echinometra*, show a distinct muscular layer in addition to those mentioned above. The walls of the ovary of *Echinometra* never disintegrate at a touch as those of many other urchins, including *Arbacia*. No references are made to the structure of the testis wall. Fox (1923*b*) says that the muscle fibers in the walls of the gonads of *Centrechinus* are more abundant in the female than in the male, but he shows no figures.

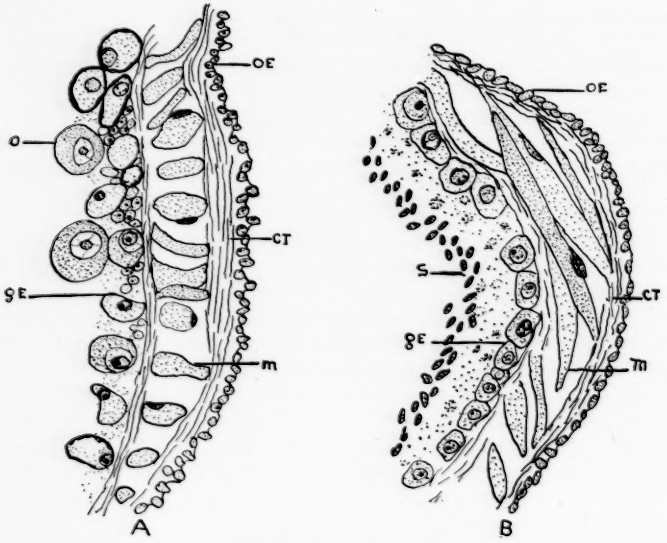

FIG. 1.—Sections of gonads of *Arbacia punctulata*. *A*, ovary showing cross-sections of muscle layer; *B*, testis showing oblique sections of muscle layer; *oe*, outer epithelium; *ct*, connective tissue; *m*, muscle layer; *ge*, germinal epithelium; *o*, maturing ovum; *s*, matured sperm.

Muscle and connective tissue may be plainly seen in the walls of the gonads of *Arbacia* when Delafield's hematoxylin and Mallory's stains are used. As illustrated in Figure 1, the wall consists of the following layers: a thin continuous band of smooth muscle cells, an inside layer of connective tissue, and the germinal epithelium. The color of the connective-tissue layers with Mallory's stain would indicate that they are elastic fibers although there is no experimental proof of this. The smooth muscle cells stain a red purple with Mallory's stain. The cells have a great power of contraction, for the length of the muscles when contracted is but a small fraction of the length when distended. The thin epithelium in contact with the muscle and connective-tissue layer is bathed directly by the perivisceral fluid, so that the contractile fibers are in a position to be readily affected by any chemical change occurring in the fluid. The cilia are not demonstrable with the technique used but are readily seen in the living material.

As is well known, the nervous system of *Arbacia* consists of a ring of nerve fibers and ganglion cells which encircles the pharynx close to the mouth, and five radial nerve trunks which lie along the entire length of the ambulacra upon the inner surface of the test and in close relation to the radial canals. The ring and radial canals are connected through the pores for the tube feet with the subepithelial nerve plexus of the ectoderm. According to Romanes (1885), this plexus serves to unite all the feet, spines, and pedicellariae. In addition, there is an internal nerve plexus which is everywhere in communication through the thickness of the shell with the external plexus. Romanes gives the function of this latter as that of bringing the feet, spines, and probably pedicellariae into relation with the great co-ordinator, the nerve band. An epithelial plexus over the intestines is also connected to the circumoral ring. MacBride (1909) says that an apical nervous system appears to be unrepresented unless an annular nerve trunk in connection with the aboral circular sinus belongs to it. Fox (1923*b*) mentions as aboral concentration of nerve fibers and cells whose fibers extend up the walls of the gonad. It was impossible with the stains used above or impregnation methods to identify nerve cells in the wall of the gonad, although using the same technique they can be demonstrated in the perivisceral epithelium.

INJURY EXPERIMENTS

There are three possibilities as to the cause of the reaction produced by injury to the urchin. If nerve fibers extend into the gonads, the stimulation to contract upon injury might be transmitted through the nerve net to the smooth muscle cells. On the other hand, the injury may release some substance which first affects the nerve cells and through them the muscles. Second, a substance capable of acting directly on the muscle cells may be produced on injury. Finally, there is the possibility that a combination of nervous and chemical stimuli may cause the shedding. Regardless of the stimulating agent it seems highly probable that we are dealing here with effects produced on smooth muscle cells.

Cutting or crushing the circumoral nerve ring or any radial band fails to cause the animal to shed its genital products. In fact, the lantern may be entirely removed by cutting through all the radial nerves and no shedding will occur provided the urchin is placed oral surface down. In an animal so treated, if the stimulus is applied carefully and few other cells injured, cuts or hard pinches may be made at any point on the radial bands or on the oral nerve ring and fail to produce shedding. An urchin may be anesthetized with $MgSO_4$ or $MgCl_2$ so that none of the customary responses to stimuli will be exhibited, yet severe injury of body tissues produces shedding in the same manner as in the untreated animal.

It may, then, be safely concluded that no injury to the central nervous system is directly involved in the reaction to injury nor is any effective stimulating substance produced by injury of nerve cells alone. Furthermore, since animals treated with $MgCl_2$ react in the normal manner to injury, the subepidermal and peritoneal nerve nets are also not directly involved.

On the other hand, it may as definitely be shown that some substance which initiates shedding is produced by injury. As stated above, the lantern may be removed from a animal by cutting around the peristome and the urchin placed oral side down in sea water and no spawning will occur. If at any time during the observation the animal is more severely injured, shedding will be induced. Immediate shedding, however, was

initiated in 92 per cent of the animals so treated when they were placed aboral side down in a watch glass. Also, in such animals placed oral surface down, uncovered with sea water, there is no shedding. Table 1 shows the result of such an experiment on 50 animals. No animal was completely spawned out and each could be made to extrude more sperm or eggs upon a more serious injury. Clearly, these results indicate the presence of some stimulating substance. It is evident that this substance moved quickly by the aid of gravity and the ciliary currents within the animal to the walls of the gonads when the animal was inverted. But, when the oral side was down, the injury substances presumably poured out of the animal and did not reach the gonads in any appreciable concentration.

Scraping the outside of the corona failed to produce shedding, but injury to the inside, either with or without removing the lantern, produced a reaction. If a blunt instrument is moved back and forth inside the corona for a short time the gonad on that side will generally shed within 10 seconds. This injury may be repeated in a new area and the gonads forced successively to shed their eggs or sperm. However, if the stimulus is severe enough in the region of one gonad, others will shed, apparently by passage of

TABLE 1

SHEDDING REACTIONS OF SEA URCHINS WITH LANTERNS REMOVED
NOT COVERED WITH WATER, OBSERVED 1 HOUR

Urchins with Oral Side Down		Urchins with Aboral Side Down	
No shedding	11 males 11 females	No shedding	2 females
		Shedding	11 females* 10 males
Slight	1 male 1 female		

*Average time interval before visible shedding: males, 56.3 seconds; females, 41.8 seconds.

the stimulating material from one to the other by ciliary activity. The following results are taken from an experiment where a blunt probe was inserted through the peristome between the radial nerves and moved with slight pressure back and forth across two interradial and one radial band. The area was stimulated for 5 seconds and the first appearance of sexual products, no matter how slight, was considered as the end point. No records were kept of sex since no appreciable difference was noted in the time interval. The time intervals before visible shedding in seconds are as follows: 3, 6, 5, 3, 15, 20, 6, 4, 4, 12, 2, 2, 3, 3, 8, 7, 4, 2, 3, 10, 6, 2, 2, 4, 7, and the average duration of the reaction was 7.32 seconds. At the end of the period of stimulation several individuals had stopped shedding. These were restimulated with the following shortened time intervals: 2, 1, 1, 1, 2, 1, 2, 1, 7, 2, 1, 4, 1, 6, and two shed immediately. The average time interval of the restimulation was 2 seconds. The difference in the two averages is probably due to the fact that it takes time for the sperm or eggs to reach the genital ducts which in the latter case were full, owing to the previous shedding. The time interval, then, is probably not a measure of the latent period for the contraction of the smooth muscle cells. From three to five such stimulations serve to empty the gonads so that further spawning cannot be induced.

That pressure on the corona is not necessary is shown by the fact that in an animal whose lantern has been removed the mere tearing out of the intestine will produce the reaction—in fact, the amount of eggs or sperm produced is so much greater and the

time of the reaction so much longer that the production of a greater amount or a different kind of stimulating substance is indicated. All the foregoing observations on the effect of injury to an animal lead to the general conclusion that the force by which the genital products are ejected and the duration of the ejaculation are directly dependent upon the amount of surface injured.

That immersion in $MgSO_4$ fails to stop this reaction is shown in Table 2. After even 5 minutes' immersion in 0.97 molar $MgSO_4$, the urchins had all the appearances of a completely narcotized animal: the tube feet were relaxed and curved or coiled, the spines were relaxed away from the aboral surface, and there was no movement. Of four which were left overnight (10 hours) in such a solution, a female shed immediately upon injury stimulation and two others in 90 and 75 seconds, respectively. A male shed only after the third stimulation 5 minutes after removal from $MgSO_4$. Similar results occur

TABLE 2*

TIME INTERVALS BEFORE VISIBLE SHEDDING PRODUCED BY INJURY AFTER IMMERSION IN ISOTONIC $MgSO_4$

(Immersion period in minutes)

5	10	20	30	60	90
12	2	2	4	3	8
8	1	1	15	45	25
9	2	3	4	15	22
5	5	1	7	17	55
7	2	2	4	4	70
3	4	3	7	.....	22
7	6	1	7	.....	.....
8	4	1	4	.....	.....
10	3	2	6	.....	.....
7	2	4	5	.....	.....

*Time intervals given in seconds.

if the urchin is immersed after the lantern is removed. That reactions occur even while the corona is full of $MgSO_4$ is not explained by the results of later experiments which show that there is an antagonism between the magnesium ion and a stimulating substance obtained from other animals. The fact that the reaction occurs in the presence of magnesium rather seems to indicate that there is some other rapidly disappearing substance formed which is acting as a stimulant and which is unaffected by the magnesium ion.

The next step was to determine if a stimulating substance could be recovered in its active form from the tissues. A method was devised whereby the gonads might be bathed by any substance soluble in the perivisceral fluid. Animals with two small slits in the peristome live indefinitely and suffer no immediate ill effects if the fluid is for the most part replaced by sea water or some other nontoxic solution. One cubic centimeter of the solution to be tested was injected into the perivisceral cavity through one slit and the excess perivisceral fluid allowed to flow out through the other. The same procedure was adapted in all the following experiments irrespective of animal size or concentration of the fluid tested.

When entire urchins are ground with a mortar and pestle and the liquid so produced

is injected into the perivisceral cavity, shedding occurs. The time interval is of the same order as that of direct injury. In order to determine from which part of the animal the substance is produced, each part was separately tested, with the following results:

Reaction	No Reaction
Chopped lantern muscle	Suspension of normally shed sperm
Chopped digestive canal	Suspension of normally shed eggs
Gonads ground with sand	Uncoagulated blood
Ground lanterns	Coagulated blood
Ground empty coronas	Heated blood
Heated sperm and eggs	
Eggs broken with glass	
Cytolyzed eggs and sperm	

The more potent extracts are obtained by heating over a water bath, but heating longer than 30 minutes does not increase the potency. Isotonic sodium chloride causes no reaction in the presence of other ions and consequently is used to dilute the extracts. If the ground tissue is heated with sodium chloride, it is a more powerful stimulant than if the chloride is added after heating. Based on the amount of dilution possible, the potency of the solutions obtained from different structures in one series of experiments

TABLE 3*

EFFECTIVE EXTRACTS OBTAINED FROM COMPARATIVE AMOUNTS OF *Arbacia* TISSUE, HEATED OVER A WATER BATH

Tissue	Number Tested	Average Time Interval Period	Average Duration of Reaction	Average Pores Involved	Dilution Possible
1 gm. lantern muscles...	4	6.2	90	2.2	none
1 gm. digestive canal...	5	5.6	68	1.4	none
5 gm. testes.....	10	7.5	.....	3.2	1–5
5 gm. ovaries.....	10	6.6	.....	3.0	1–5
5 cc. concentrated sperm	10	5.6	168	2.7	1–5
5 cc. concentrated eggs..	10	5.8	198	2.5	1–4
1 empty corona.....	4	7.0	135	2.0	none
1 whole animal unshed..	10	6.1	140	3.4	1–0.5
1 whole animal shed....	10	7.1	60	1.4	none
5 gm. lanterns.....	4	5.6	36	1.7	none

* Time intervals given in seconds.

is shown in Table 3. The soft tissues were ground with sand; those where calcareous plates were present, without sand. The difference in potency between the concentrated sperm and eggs or the gonads and the other tissues might be due rather to the presence of more concentrated cells than that an especially potent extract is found in these structures.

The gonad walls of an injured urchin may be seen to contract and push the genital products out, so that there is no doubt but that the contractile fibers of the gonad walls are involved in the shedding reaction. The contraction moves from the tips of the tubules toward the genital duct and forces the products outside. The time interval

noted above is the result of the time required for the eggs or sperm to reach the tube as well as the latent period of contraction of the smooth muscle cells. If a drop of potent extract is dropped directly on a gonad, immediate contraction of the wall in that region is apparent and the length of time until the products are seen to pass out of the duct is of the same order of magnitude as the time interval after injection. In a previously stimulated gonad the visible ejection of products from the genital duct is almost immediate because of the fact that the duct is already full.

The effective principle in the extract passes through ordinary filters and even through collodion and cellophane. It is not affected by heating to boiling or by freezing. It is effective after being kept without preservatives for two months. Within the pH range of 5–8.2, no difference in the time interval or the mode of reaction could be detected. Concentrations below pH 5 cannot be tested, for sea water alone with a pH below 5 will cause contraction of the walls and ejaculation of the products. The evidence seems, then, to indicate that the stimulating substance is independent of any normal changes in pH. The substance is not specific in stimulating the gonads of *Arbacia*, for both *Asterias* and *Echinarachnius* will shed, when stimulated, with the extract of *Arbacia*.

Not only is isotonic $MgCl_2$ ineffective when injected alone, but it prevents or antagonizes the activity of the extract. Tissues extracted in the foregoing manner with $MgCl_2$ instead of isotonic sodium chloride fail to cause the reaction, and even active tissue extract is rendered inactive by addition of sufficient $MgCl_2$. An extract which is active when diluted in the proportion of 2 parts NaCl to 1 of extract will be inactive when diluted by an equal amount of isotonic $MgCl_2$.

A stimulating substance with the same properties may be obtained from a number of other animals. Whole *Echinarachnius* and *Asterias*, ground with a mortar and pestle, produce substances which stimulate the gonad walls of *Arbacia*. The muscles of the frog and the sea minnow (*Fundulus*) are also effective sources of stimulating substances. Table 4 shows the results of a series of experiments in which the muscles of the frog and of the minnow were heated in sodium chloride over a water bath after having been ground with sand. These extracts are dialyzable and may not be filtered out, and are effective after having stood without preservatives for two months. They are also antagonized by $MgCl_2$ in the same manner as the *Arbacia* extracts.

IONIC EXPERIMENTS

Sea water itself does not produce a contraction when washed over the gonad walls, nor does isotonic NaCl or $MgCl_2$. Sodium chloride under certain conditions may cause a contraction. If the perivisceral cavity of an animal is washed many times with NaCl, eventually the reaction may occur, in consequence either to the onset of death or of the toxic nature of the ions. However, it is possible for an animal with an intact perivisceral cavity to be immersed for several hours in NaCl without suffering any permanent ill effects. Spawning may occur spontaneously after such a long immersion. If the lantern is removed and all the blood carefully washed out, shedding occurs when immersed in isotonic NaCl but may be prevented by the addition of 5 per cent isotonic $CaCl_2$. No amount of washing with this mixture can produce a reaction. If, however, enough isotonic KCl is added to make the solution 0.0080 molar for potassium, the reaction will start. It can again be stopped by adding enough isotonic $MgCl_2$ to make the solution 0.025 molar for the magnesium salt.

Using the method described for the extract, the effect of calcium and potassium was

studied. Isotonic chloride salts were used. When potassium chloride is dropped directly on the gonad, a visible steady contraction of the gonad wall occurs, so, when the potassium salt is injected, a steady powerful stream of genital products comes from all five pores. Since, unless the viscera are repeatedly washed with NaCl, this salt never pro-

TABLE 4

TIME INTERVAL BEFORE VISIBLE SHEDDING AND POSSIBLE DILUTIONS OF EXTRACTS OBTAINED FROM FROG AND *Fundulus* MUSCLE

Muscle	Isotonic NaCl per Gram (Cc.)	Average Time Interval of 5 Arbacia (Secs.)	Possible Dilution
Frog	3	7.2	1 ex. 1 Na.
Frog	5	5.4	no dilution
Frog	2	6.3	1 ex. 1 Na.
Frog	5	6.1	no dilution
Fundulus	15	7.8	1 ex. 2 Na.
Fundulus	14	6.7	1 ex. 1 Na.

duces a contraction, sodium was used to dilute the potassium. Concentrations as low as 0.005 molar for potassium chloride in sodium chloride produce shedding. The time intervals before visible shedding are relatively independent of the concentration or seem to show a slight increase in the lower concentrations. They average under 10 seconds for the range of 0.5 to 0.005 molar. They are also independent of sex. Duration of the period of spawning is directly dependent on the concentration. Table 5 illustrates these

TABLE 5*

RESULTS OF INJECTION OF POTASSIUM CHLORIDE DILUTED WITH SODIUM CHLORIDE

Molarity of KCl	Average Time Intervals (Secs.)	Average Duration of Spawning (Secs.)	Pores Involved
0.50	2.5	276.0	5.0
0.10	3.1	192.0	5.0
0.050	4.2	29.6	1.7
0.010	5.0	16.4	1.4
0.0050	7.2	6.2	1.3

* Twenty-five animals used for each concentration.

points. The reaction is absolutely certain to occur for the concentrations of 0.5, 0.1, and 0.05 molar, but depends in the last two concentrations on the physiological condition of the animal. To illustrate, during the period when many *Arbacia* are normally shedding, all the foregoing concentrations are effective, but during the early part of the summer from 33 to 50 per cent of the animals tested failed to show the reaction when stimulated by a concentration lower than 0.02 molar. Other potassium salts are also effective in producing the contraction of the gonad walls.

Potassium chloride may be rendered inactive by the presence of magnesium chloride or magnesium sulphate. If the KCl is diluted with isotonic $MgCl_2$, the urchins when stimulated will react for shorter periods of time until, with a dilution of 1 part of isotonic KCl to 7 parts of isotonic $MgCl_2$, there is no reaction. In fact, magnesium-free sea water is capable of inducing contraction of the smooth muscle cells. Herbst's (1897) sea water contains 1 part KCl to 7.5 parts $MgCl_2$. Using Herbst's formula but replacing the magnesium with sodium, tests were made. Five males shed after a single injection and three females after a second injection. McClendon's (1916) sea water with a proportion of 1 part KCl to 5.5 parts of $MgCl_2$ caused a reaction in all animals tested, when made up without magnesium.

Calcium chloride causes a different sort of contraction. When a drop of isotonic $CaCl_2$ is placed on the gonad, the walls may be seen to contract and relax in a rhythmical

TABLE 6*

SHEDDING REACTION PRODUCED BY ISOTONIC CALCIUM CHLORIDE

Time Interval	Rhythm	Second Application of $CaCl_2$	KCl Added
60	12	no reaction	no reaction
0	9	slight	no reaction
42	11	slight	slight
42	.....	no reaction	no reaction
126	8	no reaction	no reaction
25	.....	no reaction	no reaction
41	12	slight	slight
48	13	fair	slight
82	12	no reaction	no reaction
46	12	no reaction	no reaction
7	8	no reaction	no reaction
54	10	no reaction	no reaction

* Time intervals are the averages in seconds of the five pores while rhythm is given in spurts per minute.

manner. Injection of 1 cc. of isotonic $CaCl_2$ produces this characteristic rhythmical shedding and completely empties the gonads of their genital products. Table 6 gives the results of the experiments on injection of calcium. Calcium chloride is effective when diluted with isotonic sodium chloride in a proportion of 1 part calcium to 4 sodium, but fails to be effective in greater dilutions. There is a definite relationship between the concentration of calcium and the time interval before visible shedding and between the concentration and the duration of the shedding reaction. Also in all cases the time interval is longer for females than for males. The average time intervals are shown in Table 7.

A few animals will react in the higher dilutions, but the time intervals are from 8 to 20 minutes. Two parts of magnesium chloride to 1 part of calcium chloride will prevent the reaction upon injection. Animals exposed to magnesium chloride inside and outside for 12 hours showed the normal reaction and latent period when stimulated with potassium chloride, but failed to show any reaction with calcium chloride. Even after being

removed to sea water for 6 hours they still failed to show any reaction when stimulated with calcium.

When calcium and potassium chlorides were mixed, the reaction seemed to be brought about by an addition of their effects rather than the reaction of one or the other. In the foregoing experiments with artificial sea water a higher percentage of reaction occurred when the calcium remained in the solution than when both calcium and magnesium were replaced by sodium. One part of potassium chloride to 8 parts of calcium chloride produces a characteristic potassium reaction, steady jets of genital products at short time intervals. One part of potassium to 9 parts of calcium produces a percentage of rhythm —at first all the jets were steady, but later rhythm developed in all animals. One part potassium to 10 parts calcium produces rhythm, and the time interval is the longer one characteristic of the calcium salts.

The foregoing experiments conclusively demonstrate that the contractile tissues of the walls of the gonads are definitely affected by the presence of abnormal amounts of the common ions of sea water in the perivisceral fluid around them. Bethe (1929) has shown that *Arbacia* tissues are permeable to ions in the surrounding medium. His findings are further substantiated by the fact that immersion in solutions of these salts as well as the extracts will produce shedding. Solutions are made up in sea water so dilute that the osmotic changes are not a factor. Table 8 shows the results of exposing normal uninjured sea urchins to such solutions. Controls in the same amount of sea water with equivalent amounts of isotonic sodium chloride added and subject to the same temperature changes were carried along with all these animals. No spawning occurred in any control. These experiments furnish further evidence that more than one kind of muscle-stimulating substance is present in the extract. That long exposure to low concentrations of extract initiates shedding indicates that certainly some other factor than calcium or potassium is present in the extract.

TABLE 7

AVERAGE TIME INTERVALS FOR INJECTION OF ISOTONIC AND DILUTED CALCIUM CHLORIDE (DILUTED WITH NACl)

Arbacia Injected	0.3 Molar $CaCl_2$ (Secs.)	0.2 Molar $CaCl_2$ (Secs.)	0.1 Molar $CaCl_2$ (Sécs.)
Males (24).....	18	60	84
Females 28).....	95	180	140

Extracts, isotonic potassium chloride, and calcium chloride placed on the aboral surface of an unimmersed urchin will also produce shedding. Calcium alone produces a reaction in from 20 to 90 seconds and potassium alone in from 15 to 45 seconds. On the other hand, if potassium chloride follows calcium within 2 minutes, the reaction occurs in from 3 to 10 seconds and is much more general. Of 70 animals tested, all were forced to spawn by adding 3 drops either of isotonic potassium chloride or of isotonic calcium chloride, or of both, to their aboral surfaces. Twelve of them required a second application. Extract of *Fundulus* muscle (2 gm. to 20 cc. of NaCl) produced a reaction in this manner as did extract of frog muscle and whole *Arbacia*. Since the surface is wet there

is undoubtedly a penetration of ions and of the stimulating substance of the extract through the permeable wall of the urchin.

The effect of many other substances was tested on the walls of the gonads. Some of them produced contraction and others did not. Among those which produced contraction to a greater or lesser extent are alcohol (below 5 per cent), nicotine, 0.5 molar

TABLE 8

RESULTS OF IMMERSION OF URCHINS IN SEA WATER CONTAINING STIMULATING SUBSTANCES

Stimulating Substance	Dilution in Sea Water	No. Tested	No. Showing Reaction	Time Interval Under (Hrs.)
Isotonic KCl	1–30	14	4	3
			10	8
	1–75	9	2	1½
Isotonic $CaCl_2$	1– 30	11	11	1
	1– 50	15	7	1
			4	1½
			4	4½
	1– 75	8	3	1
			2	1½
			3	2
	1–100	6	3	7
			1	8
			2	9
Isotonic $CaCl_2$–KCl	1–1–30	20	12	3
			8	4
	1–1–50	10	10	4
Undiluted whole *Arbacia* extract	1–2500	5	5	2
	1–3700	4	4	2
	1–7500	17	17	2
	1–15,000	4	4	2
	1–75,000	13	13	4

strontium chloride, chloroform, and ether. On the other hand, high alcohol concentrations (above 5 per cent) isotonic sucrose or glucose, veratrin, adrenaline, and chloretone failed to produce a reaction. The consideration of these tests may be discussed in a later paper.

DISCUSSION

The solution of the problem as to why injury produces shedding resolves itself into an analysis of substances capable of stimulating smooth muscle cells. The gonads are stimulated to contract by substances produced at the point of injury of tissues within

the animal, by extracts of *Arbacia* tissue, by extracts of vertebrate muscle, by calcium, and by potassium salts. Of these substances, all are antagonized by magnesium except substances produced at the point of injury within the animal. The proportion of $MgCl_2$ required to antagonize the extracts and potassium chloride is about the same, but much less is required to prevent the effect of calcium. Animals anesthetized by magnesium may readily be stimulated by potassium, by injury, or by extracts, but cannot be stimulated by calcium.

The evidence suggests that, immediately after injury, a humoral substance, more potent than the extract, is present. The following observations tend to illustrate this point, that in addition to the stimulating substance in the extracts other muscle-stimulating substances exert a momentary effect. If one takes all the liquid produced by scraping a small portion of corona and injects it into an animal, the reaction occurs with noticeably less force and completeness than the reaction produced by injury to the same area within the animal. Again, it is possible to make all five gonads shed strongly when the intestine is torn out of an animal whose lantern is removed. Such a reaction is not obtained even with the injection of undiluted extract. The intestine may be torn out while the animal is full of magnesium chloride and shedding occurs normally. In addition, the urchin may be stimulated by direct injury while the corona is full of magnesium chloride. There is no doubt but that magnesium antagonizes the stimulating principle in the extracts prepared from other animals, but it may not antagonize some of the other substances which may be temporarily present. Another possibility, which must not be overlooked, is that the stimulating principle of the extract is produced in such large amounts at the time of injury that magnesium cannot prevent its effect.

Tissue extracts are known to contain a number of substances capable of producing contraction in muscle fibers (Barker, 1935; Craven and McCrea, 1934). Of these, all but potassium salts are organic and for the most part unstable. According to Best and McHenry (1931), histamine possesses a number of characteristics similar to those of the stimulating principle of the extracts obtained in these extractions. Histamine is of almost universal occurrence, will survive for long periods at different temperatures, and may be extracted by water, dilute alcohol, or boiling HCl. It is, however, rapidly destroyed by histaminase in injured tissues and is unable to withstand bacterial action. Histamine or some similar substance might be formed in effective amounts at the time of injury as well as the more stable principle of the extracts.

Acetylcholine and choline found in most tissues also rapidly disappear unless preserved by alcohol or trichloroacetic acid. Bacq (1935) believes that cholinergic nerves exist in echinoderms, although only traces of acetylcholine may be detected. The presence of such nerves might well be a factor in the immediate effect of injury. Such nerves stimulated by injury would release the effective humoral substance even in the presence of magnesium.

Considering now the extracts obtained from ground tissues, it seems impossible that the stimulating principle is one of the organic compounds of muscles. However, the possibility that it might be potassium must not be overlooked. That it is not one of these unstable organic compounds seems particularly likely when it is realized that the extracts of this experiment are effective after being kept for two months without a preservative. Potassium salts, on the other hand, are very stable and exist in the cells of most tissues, especially muscles, in appreciable amounts. It is possible, according to Fenn and Cobb (1934, 1935), to remove a high percentage of the potassium

salts from frog sartorius muscles. Even uninjured cells under the conditions of this experiment lose 70 mg. per cent of their potassium into the water surrounding the cells. Using Fenn's figures it is possible to calculate the molarity of the potassium released by frog muscles. It was found to be within the range of molarities capable of stimulating the gonad walls. No analyses have been made of the amount of potassium in the tissues of *Arbacia*. There are other similarities between potassium and the extract. The reaction is of the same nature whether produced by potassium or by the extract. There is a strong tonic nonrhythmical contraction which may in both cases be antagonized by the presence of the same proportion of magnesium. Extracts placed on the exterior produce the same type of spine and tube feet movement as potassium does.

There are, on the other hand, significant differences between the action of potassium and the extract. Both produce shedding when the animals are immersed in dilute solutions but the extract is not only more generally effective but is effective in much lower concentrations (Table 8). Immersion in the concentrations of potassium used may be too toxic to produce a normal effect. Future experiments using more dilute potassium chloride would clarify this point. Compared to potassium, calcium shows a greater effectiveness in producing a reaction by immersion than by injection (Tables 5, 6, 8). Calcium certainly is a less toxic ion and its effectiveness upon immersion may be explained by the fact that there is an exposure to a more constant concentration over a longer period of time with less toxicity to the urchin. Generalizations concerning the stimulating principle at this point are dangerous, for we have no direct proof that we are dealing with the same substance even in the different extracts. It can be said, however, that the stimulating properties of the extracts seem to be more like those of potassium than like any other known constituent of tissues.

Information concerning the action of various ions on muscle tissue is in a chaotic condition chiefly because of the fact that there is no knowledge as to how these ions exert their effect. There is less information concerning invertebrate muscle than vertebrate and examples are even fewer for invertebrate smooth muscle. Wells (1928) made a study of the effects of ions on the crop of *Aplysia* and *Helix*, and on the heart of *Maia*. According to him, calcium is a depressant when moderately in excess and its lack causes a contraction. Potassium causes a contraction of the crop both when it is added and when it is removed. Many other investigators have reported that potassium causes contraction of both smooth and striated muscles. Höber (1927) found a large contracture of the smooth muscle of the sea cucumber in isotonic potassium chloride. Chromatophores of fishes, according to Spaeth (1913), contract in an excess of potassium, sodium, and magnesium. Zoethout (1902) observed contraction of the gastrocnemius bathed by potassium, a reaction which was antagonized by calcium. It may safely be concluded that there is nothing unusual in the fact that potassium produces contraction of the smooth muscle cells of *Arbacia*.

It is also fairly well known that many muscle systems react in the same way to both potassium and calcium. Among the investigators who have observed such contraction are Bacq and Rosenbluth (1934) in the denervated nictitating membrance and uterus of the cat, Tate and Clark (1922) in the uterus of the rabbit and cat, Fienga (1910) in chicken esophagus and frog muscle, and Cantel (1929) in the frog's stomach. That even similar systems do not react in the same way is shown in the work of Tate and Clark. Rat and guinea-pig uteri contract in potassium but relax in calcium. There are some differences between the two salts in *Arbacia*. Calcium is slower to act than potassium

and produces a rhythmical contraction rather than a steady one. The stimulation of the muscle by potassium seems an all-or-none reaction, since the time interval is independent of the concentration. With calcium, however, there is a definite relationship between the concentration and the time interval before visible contraction. In addition, there is a sex difference with calcium. Both calcium and potassium salts are antagonized by magnesium. It is possible that, *in Arbacia*, potassium and calcium are affecting different parts of the gonad wall, each of which is affected in some way by magnesium. It is more probable, however, that the relation is more complex and cannot be explained without a clearer knowledge of how these ions penetrate.

SUMMARY

1. A substance capable of initiating the shedding of genital products is produced by injury of the body tissues of *Arbacia*.

2. An aqueous extract capable of inducing shedding is obtained by crushing and heating tissues of *Arbacia*.

3. A substance similar in action may be obtained from crushed *Fundulus* or frog muscle and from ground *Asterias*, *Echinarachnius*, and *Ostrea*.

4. Calcium chloride and potassium chloride induce the shedding reaction.

5. The extract, calcium chloride, and potassium chloride are antagonized in their activity by magnesium chloride and magnesium sulphate. A stimulating principle formed by direct injury is, however, effective in the presence of excess magnesium.

6. The possibility that the stimulation to shed is due partly to the presence of potassium and partly to the presence of some organic substances in the extract is considered.

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NUTRITIONAL STUDIES OF THE WEBBING CLOTHES MOTH TINEOLA BISSELLIELLA HUM.

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SINCE a complete knowledge of the nutritional requirements of an insect is an important tool in studying economic control, and since the peculiar feeding habits of the clothes moth render it an object of interest to the student of nutrition, it was considered worth while to undertake a detailed study of the effect of various food factors upon the life-cycle of the webbing clothes moth, *Tineola bisselliella*. Furthermore, such studies are of value from the point of view of evolution. Material which will contribute to the fund of information regarding the food requirements of representatives of the various phyla is sought by the general biologist and the comparative physiologist, as well as by students in the more specialized fields of economic entomology or nutrition.

The general plan of the experiments undertaken has been a determination of the effect of various constituents of the diet on the duration of the life-cycle, in days. The life-cycle of an insect is defined as the time elapsing from the laying of the egg to the emergence of the adult insect. Such an expression of results rests on the assumption that an optimum diet allows development in a minimum of time. A similar technique has been used for Tribolium studies (Sweetman and Palmer, 1928) and for studies on the Mediterranean flour moth (Richardson, 1926).

Physiological or nutritional studies upon cold-blooded animals must be rigidly controlled in regard to physical environment. The need for careful regulation of temperature, humidity, and consistency of the diet is particularly important where the animal lives surrounded by its food. Such animals have never had to adapt themselves to rapid changes in temperature and humidity. Attempts have been made in these experiments on clothes-moth larvae so to regulate other conditions that dietary constituents remain the only variables.

Diets were brought to a finely divided condition by grinding through the ½-mm. screen of a Wiley mill or by pulverizing them in a ball mill. Diets of more than one constituent were mixed by grinding with a mortar and pestle. The diet to be tested was sampled into shell vials of 20×40 or ×80 mm. Newly hatched larvae were then transferred to the vials by means of a camel's-hair brush. All larvae were examined under a binocular after transfer, to eliminate any injured animals. Cotton plugs or very fine muslin squares were used to cover the vials and prevent the escape of the larvae. Thirty animals were used in most experiments. The number of larvae per vial varied from one to five, but three individuals per vial were finally selected as the most satisfactory number.

The temperature was maintained at 25° C. in a thermostatically controlled incubator. The humidity was roughly controlled by the presence of a dish of water in the incubator. In certain of the experiments a relative humidity of 75 per cent (Griswold and Crowell, 1936) was maintained by keeping the vials in museum jars over a saturated solution of sodium chloride.

The vials were examined weekly for five weeks, and thereafter were examined daily for adult moths. Records were kept of the date on which the eggs were laid and the date of emergence of the adult moths.

EXPERIMENTAL STUDIES

Fish meal has been found the most satisfactory substance in which to rear clothes moths (Griswold, 1933). The consistent results obtained with this food show that the variations in length of life-cycle on other diets are comparable with the variations experienced in the feeding of incomplete diets to higher animals (Sherman and Spohn, 1923). A control group on fish meal was conducted simultaneously with the test diets. The results from these control experiments have been summarized in Table 1 to show that concordant results may be expected from repetition of experiments. Apparently, there is no seasonal variation in the length of the life-cycle of the clothes moth when conditions of food, temperature, and humidity are controlled. Experiments 10–29, inclusive, were conducted at 25° C. and at the humidity of the incubator, while Experiment 42 was conducted at a temperature of 25° C. and at 75 per cent relative humidity.

The fish meal was found to contain 8.49 per cent moisture at the humidity of the incubator and 12.01 per cent moisture at 75 per cent relative humidity. From Table 1 it may be seen that this range of moisture content has little effect on the life-cycle.

TABLE 1

CONTROL EXPERIMENTS WITH FISH MEAL

Diet No.	Date Eggs Were Laid	Number Started	Number Emerged	Average Life-Cycle in Days
10	Aug. 31—Sept. 1	30	28	56.8±1.90
12*b*	Sept. 27–28	30	28	50.5±1.69
18	Sept. 27–28	30	28	48.3±0.28
15	Feb. 8–9	30	28	52.7±1.02
29	Nov. 21–22	30	29	55.0±2.49
42	June 7–8	30	23	48.2±0.55

Other experiments (Griswold and Crowell, 1936) have shown the effect of more extreme ranges of humidity on the development of *T. bisselliella*.

It was hoped that a diet of fairly pure known constituents could be found—in other words, a "purified diet" which would supply all essentials and permit the emergence of the adult moth in as short a time as would fish meal. Casein has been listed (Back and Cotton, 1931) as one of the products infested by clothes moths. This served as a starting-point for a purified diet. A supply of commercial casein which had been rendered vitamin-A-free served as a basal diet (diet No. 21). The beneficial effects on clothes-moth larvae of yeast, when added to a basal diet of wool, had been reported (Colman, 1932). Accordingly, the basal diet (No. 21) was supplemented with 5 per cent yeast for diet No. 22. Since commercial casein is known to carry small amounts of vitamin B as well as vitamin A, a purified A- and B-free casein was prepared by extracting the commercial casein with dilute acetic acid and then with alcohols of increasing strength (Hawk and Bergeim, 1931). This new product served as diet No. 27. Since the addition of large amounts of yeast may affect the protein quality of the diet, a more potent source of the vitamin-B complex was provided in the Harris B concentrate in diet No. 30. The data from these experiments appear in Table 2.

The results are clearly defined. Vitamin-A-free casein permits some growth and development. The addition of 5 per cent yeast to commercial casein decreases the mortal-

ity and increases the rate of development. Vitamin-A- and B-free casein alone will not support growth or development. The addition of 0.5 per cent of Harris B concentrate allows for some growth and development. This serves as another example of the requirement for part or all of the vitamin-B comlpex throughout the animal kingdom. None of these nutritive media compares with fish meal for low mortality or short life-cycle.

To determine whether the protein quality or the mineral content of diet No. 30 might be at fault, other experiments were set up to eliminate possible deficiencies. The source of the protein of diets No. 36 and No. 41 was a combination of casein and lactalbumin. Both products had been purified to remove vitamins A and B. Minerals were supplied in diets No. 31 and No. 36 as ash of fish meal. The results of diets No. 29 and No. 30 from Table 2 have been repeated in Table 3 for comparison.

The results obtained with diets No. 36 and No. 40, when compared with diets No. 30 and No. 31, demonstrate clearly that casein does not supply optimum amounts of the amino acids required for the growth and development of *Tineola*. The addition of lactal-

TABLE 2

CASEIN AS A PURIFIED DIET

Diet No.	Diet	Percentage Composition	Number Started	Number Emerged	Average Life-Cycle in Days
21	Vitamin-A-free casein	100	30	12	122.9± 8.87
22	Vitamin-A-free casein Yeast	95 5	30	25	82.9± 2.56
27	Vitamin-A- and B-free casein	100	30	.....	.....
30	Vitamin-A- and B-free casein Harris B concentrate	99.5 0.5	30	18	128.6±11.52
29	Fish meal	100	30	29	55.0± 2.49

bumin to the casein provides a complete source of protein. No explanation can be given for the high mortality on diet No. 40. In all other cases short life-cycles have been accompanied by low mortality. In Experiments 36 and 40 we are dealing with life-cycles comparable to those obtained with fish meal. These diets may be of use as important tools in further studies of the nutrition of the clothes moth.

Wool, a common diet of clothes moths, is rich in cystine. Since casein is notably deficient in cystine (Osborne and Mendel, 1912) and since it has been shown (Michelbacher, Hoskins, and Herms, 1932) that highly purified casein is too low in cystine content for the development of *Lucilia*, diet No. 37 was set up in comparison with diets No. 31 and No. 36. The cystine used was obtained from the Pfanstiehl Chemical Company. From the results it is apparent that cystine is not the only factor supplied by the lactalbumin. Nor does the addition of cystine markedly improve the casein.

Comparison of diet No. 30 with No. 31 and of diet No. 36 with No. 40 indicates that the mineral requirement of the clothes moth is very low. The addition of fish-meal ash to an otherwise complete food seems to have neither a beneficial nor a detrimental effect.

Since clothes moths are commonly considered as pests consuming woolen materials, it was deemed advisable to conduct a few experiments using sheep's wool as a basal diet.

These experiments were conducted at 25° C. and at 75 per cent relative humidity. One diet (No. 45) consisted of unwashed wool. This wool was selected as being as free from foreign material as possible. Diet No. 46 consisted of wool which had been thoroughly

TABLE 3

MORE COMPLETE PURIFIED DIETS

Diet No.	Diet	Percentage Composition	Number Started	Number Emerged	Average Life-Cycle in Days
29	Fish meal	100	30	29	55.0± 2.49
30	Vitamin-A- and B-free casein Harris B concentrate	99.5 0.5	30	18	128.6±11.52
31	Vitamin-A- and B-free casein Fish-meal ash Harris B concentrate	94.5 5.0 0.5	30	22	161.1± 9.52
36	Vitamin-A- and B-free casein Vitamin-A- and B-free lactalbumin Fish-meal ash Harris B concentrate	84.5 10.0 5.0 0.5	30	30	57.9± 0.53
37	Vitamin-A- and B-free casein Fish-meal ash Harris B concentrate Cystine	94.0 5.0 0.5 0.5	30	25	122.6± 5.7
40	Vitamin-A- and B-free casein Vitamin-A- and B-free lactalbumin Harris B concentrate	89.5 10.0 0.5	30	20	48.2± 0.20
41	Vitamin-A- and B-free casein Vitamin-A- and B-free lactalbumin	90 10	30	0	.....

TABLE 4

WOOL AS A BASAL DIET

Diet No.	Diet	Number Started	Number Recovered	Age in Days	Average Weight in Milligrams
45	Unwashed sheep's wool	30	1	67	1.2
46	Washed sheep's wool	30	10	67	0.33
48	Washed sheep's wool (99.5%) Harris B concentrate (0.5%)	30	19	52	2.18

washed with soap and warm water, rinsed in tap and distilled water, and dried at room temperature. Diet No. 48 was made up of diet No. 46 with the addition of 0.5 per cent Harris B concentrate.

When the experiments were set up, it was though that diet No. 45 would serve as a positive control, but this did not prove to be the case. Experiment 48 was started 2 weeks later than Experiments 45 and 46. All three experiments were stopped when

Experiment 48 had been in progress for 52 days. The larvae were removed and weighed. The results are to be found in Table 4.

The weights and number recovered are based only on live larvae. Several dead larvae were found in the vials in all cases. These data certainly serve to show the deficiency of the wool used in vitamin B. The failure of the untreated wool was rather surprising, but it probably lacked sufficient contamination.

It may be observed that all the purified diets used in the foregoing experiments were free from fat and the fat-soluble vitamins. An experiment was also conducted using defatted fish meal as a diet. An average life-cycle of 56.5 ± 1.06 days was obtained. This compares very favorably with the life-cycles obtained on untreated fish meal, and supplies further evidence that fat is not required by clothes-moth larvae. There is a common belief that clothes-moth larvae, in seeking soiled spots of woolens, are seeking a source of fat. It may now be postulated that they are seeking a source of the vitamin-B complex. Various workers (McCollum and Simmonds, 1927) have demonstrated the presence of vitamin B in urine and feces. The question arises as to the possible vitamin-B content of perspiration.

CONCLUSIONS

The nutritional requirements of the webbing clothes moth, in spite of its peculiar feeding habits in nature, may be satisfied by a purified diet consisting of casein, lactalbumin, Harris B concentrate, and fish-meal ash. The value of the fish-meal ash is questionable.

Part, or all, of the vitamin-B complex is essential for the growth and development of *T. bisselliella*.

The clothes moth requires neither fat nor the fat-soluble vitamins.

Purified casein is not an adequate protein for the optimum development of the larvae of *Tineola*. Purified lactallbumin suppements the casein satisfactorily. The addition of cystine to the casein does not markedly improve its quality, as far as *Tineola* is concerned.

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